GLYCAN CHAIN STRUCTURE AND FUNCTION OF ANURAN EGG JELLY COAT ACIDIC GLYCOPROTEINS

カエル卵ゼリー層酸性糖タンパク質の糖鎖構造と機能

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学 位 論 文

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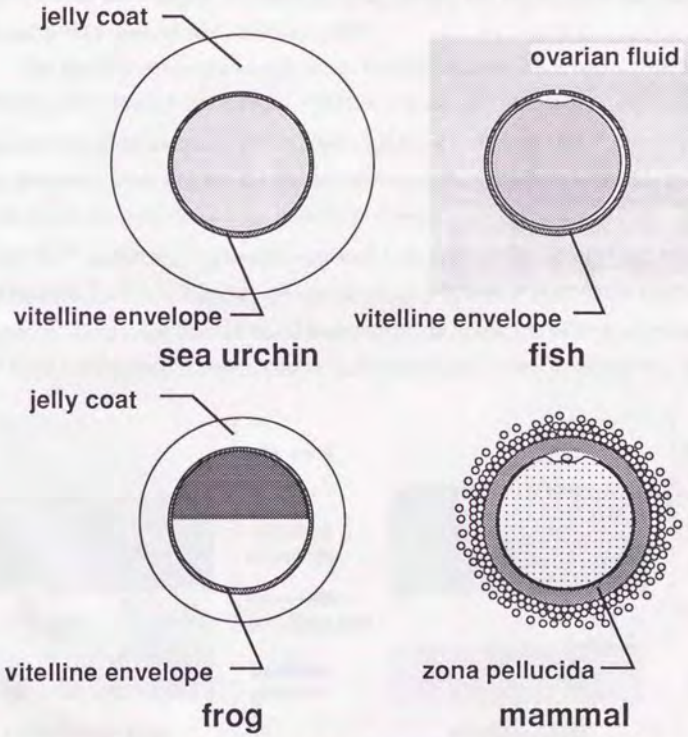
CONTENTS

ABBREVIATIONS

BSA :	bovine serum albumin
CGL :	cortical granule lectin
COSY :	correlated spectroscopy
EDTA :	ethylenediaminetetraacetic acid
ELISA :	enzyme-linked immunosorbent assay
FAB-MS :	fast atom bombardment mass spectrometry
GalNAcol :	*N*-acetylgalactosaminitol
GLC :	gas liquid chromatography
Hex :	hexose
HexNAc :	*N*-acetylhexosamine
HexNAcol :	*N*-acetylhexosaminitol
β-HexNAc'ase :	β-*N*-acetylhexosaminidase
HPLC :	high performance liquid chromatography
JGP :	major glycoprotein from egg jelly coat
B-JGP :	major glycoprotein from *Bufo japonicus* egg jelly coat
X-JGP :	major glycoprotein from *Xenopus laevis* egg jelly coat
KDN :	3-deoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid
KDN-gp :	KDN-rich glycoprotein isolated from ovarian fluid of rainbow trout
F-layer :	fertilization layer
MM :	mild methanolysis
NeuAc :	*N*-acetylneuraminic acid
NeuGc :	*N*-glycolylneuraminic acid
H-PSGP :	high molecular mass polysialoglycoprotein isolated from unfertilized eggs of rainbow trout
L-PSGP :	low molecular mass polysialoglycoprotein isolated from fertilized eggs of rainbow trout
SD :	Smith degradation
SDS :	sodium dodecyl sulfate
TBS :	Tris-buffered saline (150 mM NaCl in 10 mM Tris-HCl, pH 7.3)
TBS-T :	Tris-buffered saline containing 0.05% tween 20
TLC :	thin layer chromatography
TOCSY :	total correlation spectroscopy

INTRODUCTION

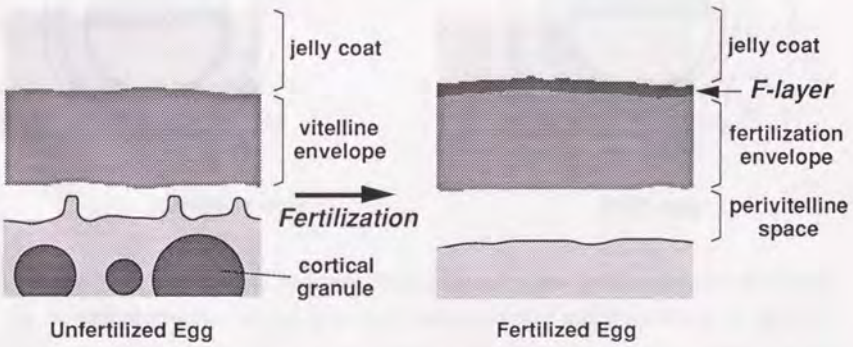
Mature eggs of most animal species are surrounded by extracellular coats or investments such as the vitelline envelope and the jelly coat (Scheme 1). These investments are the first barrier for fertilizing sperm that must be passed through before reaching the egg plasma membrane. They are also known to have multiple functions at fertilization such as recognition of homologous species, capacitation of the sperm, induction of the acrosome reaction, and prevention of polyspermy. Glycoproteins have been shown to be the major components of



Scheme 1. Egg of sea urchin, fish, frog, and mammal. Each egg is surrounded by extracellular investments such as the vitelline envelope and the jelly coat. Fish egg is spawned with ovarian fluid. Zona pellucida of mammalian egg is analogous to vitelline envelope of other animals.

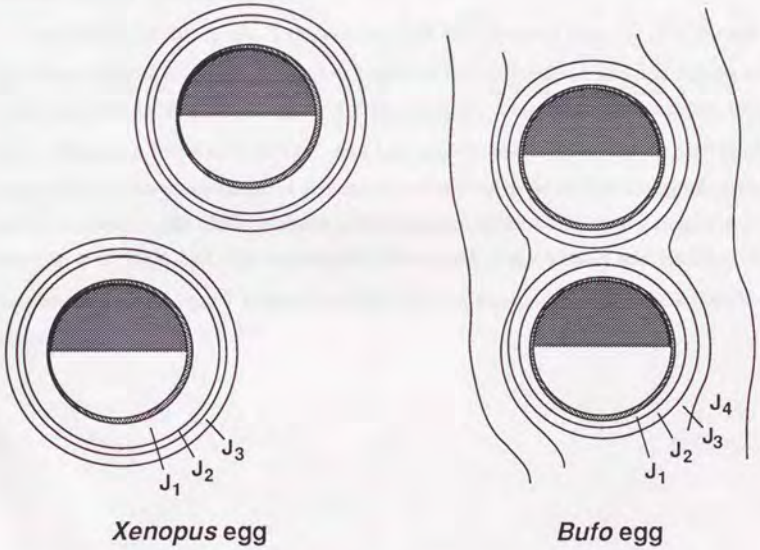
these coats in a variety of animal species of both invertebrates and vertebrates (Gwatkin, 1976; Lopo, 1983; Wassarman, 1988). Major components of mouse zona pellucida (vitelline envelope), for instance, are known to be three glycoproteins named mZP1-3, and one of them, mZP3, was shown to have activities of binding to sperm and inducing of acrosome reaction (Wassarman, 1988, 1990). In view of the prevalent occurrence of glycoproteins in the animal egg coats, the question of how these glycoproteins are involved in any function during fertilization and early development is important but remained unanswered clearly. *Bufo* and *Xenopus* egg jelly appeared to be suitable for a study to answer the question because of the abundance of the material for structural analysis and the accumulated functional studies (Katagiri, 1987; Hedrick and Nishihara, 1991).

The egg jelly coat is essential in anuran fertilization, as dejellied eggs cannot be fertilized (Elinson, 1971; Hedrick and Katagiri, 1988). It was reported that, in *Bufo*, dejellied eggs were fertilizable in media containing certain concentrations of Ca^{2+} and/or Mg^{2+} without any organic jelly materials, concluding that the primary function of the egg jelly is to provide a proper ionic environment for sperm to undergo the acrosome reaction (Ishihara et al., 1984). The divalent cation (Ca^{2+} and/or Mg^{2+}) was also suggested to be essential for *Xenopus* egg fertility (Wolf and Hedrick, 1971). In *Xenopus*, the change of egg jelly coat at fertilization was investigated in terms of fertilization layer (F-layer) formation by the interaction of the innermost jelly layer and lectin from cortical granule (Scheme 2), functioning as a block to polyspermy (Grey et al.,



Scheme 2. Change of the vitelline envelope to the fertilization envelope of anuran egg. F-layer is to form between the vitelline envelope and the innermost jelly layer by the interaction of the lectin from cortical granule and its ligand molecule.

1974; Nishihara et al., 1986). Though the layer analogous to F-layer is morphologically known not to form in *Bufo* egg jelly coat, *Bufo* egg jelly extract was reported to react with *Xenopus* CGL (Hedrick and Katagiri, 1988). The egg jelly coats are known to be composed of four layers in *Bufo* and three layers in *Xenopus* (Scheme 3), and their glycoprotein nature have been suggested based on compositional analysis (Katagiri, 1973; Yurewicz et al., 1975). But molecular bases and chemical properties of the binding of glycoproteins to calcium ion and cortical granule lectin were still unclear. The greater part of properties of egg jelly coat must be determined by major glycoproteins. Accordingly, to study function of the jelly coat, it is important that major glycoproteins are isolated and purified, and their structure and chemical properties are analyzed.



Scheme 3. Eggs of *Xenopus* and *Bufo*. Mature eggs of anuran are surrounded by jelly coat. In the case of *Xenopus*, the egg jelly coat is composed of at least three layers, i.e. J_1 to J_3, inner to outer. All the layers are concentrically surrounding egg. By contrast, *Bufo* egg jelly coat consists of four layers, J_1 to J_4, inner two layers of which surround each egg while outer two layers continuously surround the string of eggs.

The objectives of my study are twofold:

1) isolation and purification of major glycoproteins from anuran egg jelly coats, and structural analysis of their glycan chains (CHAPTER I and II);

2) analysis of calcium ion- and cortical granule lectin-binding properties of anuran egg jelly coat glycoproteins (CHAPTER III).

In CHAPTER I and II, with the aim of establishment of molecular bases of functions of egg jelly coat, I isolated and purified major glycoproteins (JGPs) from *Bufo japonicus* (CHAPTER I) and *Xenopus laevis* (CHAPTER II) egg jelly coat and determined their chemical structure. Glycan chains were released from the purified glycoproteins by alkaline borohydride treatment, and their structures were elucidated by composition analysis, methylation analysis, FAB-MS, and ^{1}H-NMR spectroscopy.

In CHAPTER III, firstly, I showed the high Ca^{2+}-binding capacity of JGPs and the importance of acidic residues (sialic acid or sulfate) in the binding by equilibrium dialysis study. I also studied the binding of fish egg surface glycoproteins containing oligo/polysialic acids to Ca^{2+}. They were found to bind Ca^{2+} with high affinity and capacity and the Ca^{2+}-binding property was the common feature of glycoproteins localized in the surface of egg and embryo. Second, by applying the ELISA method, cortical granule lectin (CGL) from *Xenopus* egg was shown to bind JGPs. ELISA analysis for JGP-related glycoproteins and modified JGPs revealed the specific ligand structure of CGL may be Fucα1$\rightarrow$2Galβ1$\rightarrow$3(GlcNAcβ1$\rightarrow$6) GalNAc.

CHAPTER I ISOLATION OF A MAJOR GLYCOPROTEIN IN *BUFO JAPONICUS* EGG JELLY COAT AND STRUCTURAL ANALYSIS OF ITS GLYCAN CHAINS

MATERIALS AND METHODS

Isolation and Purification of the Major Glycoprotein Component of the Egg Jelly Coat from Japanese Toad (Bufo japonicus) Eggs

The mature unfertilized eggs were obtained from *Bufo japonicus* purchased from the local experimental animal farm in the reproductive season (March). The eggs were placed in deionized water to allow hydration of the jelly coat, and the jelly was solubilized with De Boers solution (110 mM NaCl, 1.3 mM KCl, and 1.3 mM $CaCl_2$) containing 50 mM 2-mercaptoethanol, adjusted to pH 9.5 with NaOH (Hedrick and Katagiri, 1988). The jelly solution was separated from the eggs by decantation. After removal of insoluble materials by centrifugation at 4,000 rpm for 10 min, one-tenth volume of 90% phenol was added to the jelly solution and the mixture was stirred overnight at about 20°C. After centrifugation at 7,500 rpm for 30 min, the supernatant was dialyzed against 10 mM Tris-HCl (pH 8.0) containing 10 mM 2-mercaptoethanol. The dialyzed solution was chromatographed on a column (3.1 x 65 cm) of Whatman DE52 anion-exchange cellulose (Cl^- form). The column was eluted with a linear gradient of 0-1 M NaCl in 10 mM 2-mercaptoethanol/10 mM Tris-HCl (pH 8.0). Elution from the column was monitored by the resorcinol method (Svennerholm, 1963) for sialic acid (NeuAc used as the standard) and the phenol/sulfuric acid method (Dubois et al., 1956) for hexose (Gal used as the standard). The fractions containing carbohydrates were pooled, desalted, and applied to a Sephacryl S-500 column (2.8 x 156 cm, equilibrated and eluted with 10 mM 2-mercaptoethanol/0.1 M NaCl/10 mM Tris-HCl, pH 8.0). The elution profile was monitored by similar procedures as described above. Carbohydrate-containing fractions were pooled, dialyzed against deionized water, and lyophilized. The products were designated as B-JGP.

Alkaline Borohydride Treatment of B-JGP

B-JGP (120 mg) was incubated in 50 ml of 0.1 N NaOH/1 M $NaBH_4$ at 37°C. A half of the reaction mixture was taken out at 50 h and neutralized with 1 M acetic acid. At 110 h the rest of the reaction mixture was similarly neutralized with 1 M acetic acid.

Purification of the Liberated Oligosaccharide Alditols

The products obtained by reacting B-JGP with BH_4^-/OH^- for 50 h were desalted on a Sephadex G-25 column (2.8 x 110 cm, 5% ethanol). The pass-through fraction was fractionated by ion-exchange chromatography on a column of DEAE-Sephadex A-25 (1.5 x 40 cm, equilibrated with 10 mM Tris-HCl, pH 8.0) into the neutral fraction (N) and acidic fractions (A) which were eluted with a 0-0.4 M NaCl gradient in 10 mM Tris-HCl, pH 8.0. The elution profile was monitored by the phenol/sulfuric acid and the resorcinol methods.

(a) Neutral fraction - The neutral fraction (N) was applied to a column (1.3 x 140 cm) of Bio-Gel P-4, equilibrated and eluted with 50 mM CH_3COOH/0.11 M pyridine. The elution was monitored by the phenol/sulfuric acid method. Two peaks of neutral oligosaccharides (N-1 and N-2) were resolved. These were analyzed for carbohydrate composition and examined for homogeneity by TLC (Solvent I; see below). N-2 was further purified on a column (1.5 x 110 cm) of Sephadex G-25 (equilibrated and eluted with 10% ethanol). The eluant was monitored by the phenol/sulfuric acid method, and the carbohydrate-containing fraction was rechromatographed by the same column and the purity was examined by TLC (Solvent I). N-1 was subjected to a preparative silica gel TLC (Kieselgel 60, Merck; Solvent I). A strip from the edge was cut from each side of the plate, and the oligosaccharide was visualized with 1% orcinol in 50% H_2SO_4. The silica gel in the area corresponding to the visualized band was scraped off. The oligosaccharide was extracted from the silica gel with 20% ethanol and a small amount of silica gel was removed by passage through a Sephadex G-25 column. The N-1 sample thus purified was used for analysis.

(b) Acidic fraction - Carbohydrate-containing fractions (A) were pooled and subjected to composition analysis and TLC analysis using Solvent II (see below) after desalting on a Sephadex G-25 column (1.2 x 100 cm). The TLC analysis showed the presence of two components, which were then separated by adsorption-partition chromatography on a column of Iatrobeads 6RS 8060 (1.3 x 70 cm, pre-equilibrated and eluted with n-propyl alcohol/25% aqueous ammonium/water = 6:1:1.5). Elution was monitored by the phenol/sulfuric acid method. The fractions for which sufficient resolution of the two components was confirmed by TLC were separately pooled, and passed through a Sephadex G-25 column to remove silica gel. These two acidic glycans were denoted as A-1 and A-2, and subjected to structural analysis.

Sialidase Digestion of Acidic Oligosaccharide Alditols

The purified acidic tri- and tetrasaccharide alditols A-1 and A-2 (200 μg each as NeuAc)

were separately digested with *Arthrobacter ureafaciens* exosialidase (0.5 units, Nacalai, Kyoto) in 4 ml of 50 mM sodium acetate buffer (pH 5.5) for 24 h at 37°C. The digests were applied to a DEAE-Sephadex A-25 column (Cl^- form, 1.0 x 25 cm, equilibrated with 10 mM Tris-HCl, pH 8.0) and the pass-through (neutral) fractions were desalted on a Sephadex G-25 column. The products thus obtained were designated as asialo A-1 and asialo A-2 and used for structural studies.

Digestion of Neutral Oligosaccharide Alditol, N-1, with ß-N-Acetyl-hexosaminidase

Purified oligosaccharide alditol, N-1 (200 μg), was incubated with β-*N*-acetyl-hexosaminidase (2.5 units, Jack bean, Seikagaku Kogyo, Tokyo) at 37°C for 24 h in 0.5 ml of 0.15 M sodium phosphate buffer, pH 5.5. The digest was desalted by passing through the coupled columns of DEAE-Sephadex A-25 (0.7 x 3 cm, CH_3COO^- form) and Dowex 50W (0.7 x 3 cm, H^+ form) followed by evaporation. The sample thus obtained was referred to as d-N-1, and subjected to carbohydrate composition and methylation analyses.

Analytical Procedures

Carbohydrate composition was analyzed by GLC after methanolysis and re-*N*-acetylation as the trimethylsilyl derivatives (Nomoto et al., 1982). Sialic acid was identified by GLC after mild methanolysis in 0.05 M methanolic hydrogen chloride for 1 h at 80°C followed by trimethylsilylation (Yu and Ledeen, 1970). Methylation of oligosaccharides and GLC analysis of partially methylated alditol acetates were described previously (Iwasaki and Inoue, 1985; Nomoto et al., 1982).

Amino acids and amino sugars were determined on a Hitachi Model KLA-5 amino-acid analyzer after hydrolysis in 6 M HCl at 110°C for 24 h and 4 M HCl at 100°C for 4 h, respectively. Animo acid analysis was done by Dr. Sadako Inoue (Showa University).

TLC analysis of oligosaccharides was carried out by using silica gel plates (Merck, Darmstadt) and developing in either of the following two solvent systems: Solvent I, ethylacetate/pyridine/acetic acid/H_2O = 5:5:1:3 and Solvent II, n-propyl alcohol/25% aqueous ammonium/water = 6:1:2.5. Spots were visualized with spraying 1% orcinol-50% H_2SO_4 followed by heating at 140°C for 30 min.

400 MHz ^{1}H-NMR Spectroscopy

Oligosaccharides were treated twice with D_2O (99.85%) with an intermediate lyophilization, lyophilized, and finally dissolved in D_2O (99.95%). ^{1}H-NMR spectra were determined at 27°C with a JEOL JNM-GX400 spectrometer. The chemical shifts were expressed relative to internal sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 (the methyl proton resonance signal was set to 0 ppm). Measurements were made by Miki Nagasao (Showa University).

RESULTS

Isolation and Purification of B-JGP from the Unfertilized Eggs of Bufo japonicus

The jelly coat was solubilized by treatment of 145 g of the *Bufo japonicus* unfertilized eggs (145 ml, ~8,000 eggs) with 2-mercaptoethanol/De Boer (pH 9.5). The amount of the jelly coat before solubilization was estimated to be 65 ml from the amount (80 ml) of the dejellied eggs. The amount of high molecular mass components of the jelly coat obtained after dialysis as the lyophilized powder was 3.9 g.

Glycoprotein components in the jelly were recovered in the aqueous solution after phenol-treatment of the jelly solution. When the phenol-treated extract was subjected to Whatman DE52 chromatography in the presence of 2-mercaptoethanol, carbohydrate-containing material was eluted as a rather broad peak centered at ~0.15 M NaCl (Fig. 1.1). When this peak (see the bar in Fig. 1.1) was filtered on a Sephacryl S-500 column (1.3 x 112 cm, calibrated by use of 74.3 K and 2,000 K dextran standards, Sigma, St. Louis), marked heterogeneity was observed in the molecular mass ranging from 100 x 10^3 Da to 4,000 x 10^3 Da (Fig. 1.2). Four subfractions (referred to as fractions 4,000-2,000 kDa, 2,000-1,000 kDa, 1,000-300 kDa, and 300-100 kDa) were pooled as indicated in Fig. 1.2. To examine the nature of polydispersity, these four fractions were first analyzed for carbohydrate and amino acid compositions, but no significant difference was observed (data not shown). Second, the highest molecular mass B-JGP (4,000-2,000 kDa) was treated with 1% SDS/0.1 M NaCl/5 mM Tris-HCl (pH 8.0), and then applied to a Sephacryl S-500 column pre-equilibrated with the same solution. No change in the elution profile or elution position was observed (data not shown). Therefore, the observed heterogeneity of B-JGP was evidently a manifestation of inherent polydispersity and not of self-association phenomena. Based on these data, the four fractions were combined and used for further analysis. B-JGP consisted of 23% peptide and 77% carbohydrate (neutral sugars, 31%; hexosamines, 26%; sialic acid, 20%). Total of 1.8 g of B-JGP was recovered from 3.9 g dry weight of the egg jelly, allowing us to estimate that B-JGP was present in the jelly coat of unfertilized eggs at about 28 mg/ml concentration.

Treatment of B-JGP with Alkaline Borohydride

Two halves of the B-JGP sample were treated with 0.1 N NaOH/1 M $NaBH_4$ at 37°C for 50 h and for 110 h, respectively. Amino acid and carbohydrate compositions of B-JGP were

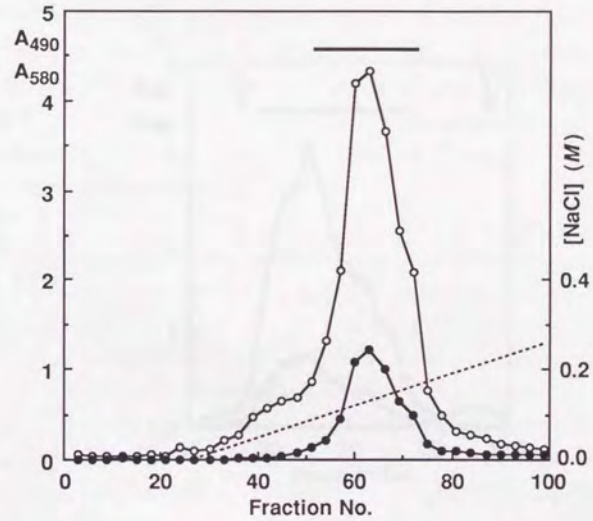


Fig. 1.1. Whatman DE52 anion-exchange chromatography of *Bufo* egg jelly coat glycoprotein. Phenol-treated extract of *Bufo* egg jelly was charged on a Whatman DE52 column (3.1 x 65 cm; Cl^- form) and eluted with a linear gradient of NaCl in 10 mM 2 mercaptoethanol/10 mM Tris-HCl (pH 8.0) from 0 (2 l) to 1.0 M (2 l) (----). 15-ml fractions were collected and analyzed for neutral sugar by the phenol/sulfuric acid method (A_{490}, ○) and for sialic acid by the resorcinol method (A_{580}, ●). Fractions eluted near 0.15 M NaCl were combined as indicated by the bar.

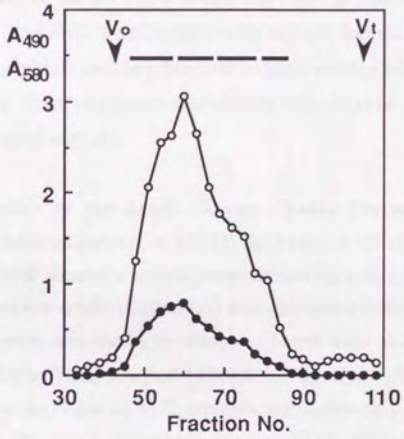


Fig. 1.2. Gel chromatography on a Sephacryl S-500 of B-JGP obtained from the unfertilized egg jelly coat of *Bufo japonicus*. The column (2.8 x 156 cm) was equilibrated and eluted with 10 mM 2-mercaptoethanol/0.1 M NaCl/10 mM Tris-HCl (pH 8.0). Elution was monitored as described in the legend to Fig. 1.1. Fractions were collected every 9 ml, and pooled as indicated by the bars. V_0 and V_t are the void and total column volumes, respectively.

analyzed before and after 50-h treatment with alkaline borohydride (Table 1.1). The serine and threonine contents decreased by 65% and 46%, respectively, and the concomitant increase in alanine and α-aminobutyric acid by the corresponding amounts was observed. The other amino acid compositions of B-JGP remained practically unchanged after 50-h treatment with alkaline borohydride reagent. The carbohydrate composition analysis of the oligosaccharide alditol products showed that about 50% of the GalNAc residue were converted to the GalNAcol residue. These results established that the glycan chains were linked through *O*-glycosidic (GalNAc) linkages to Ser/Thr. In the 110-h treated JGP, the GalNAc residue was converted into GalNAcol by 72%. However, the oligosaccharide alditols formed upon 110-h treatment were found to contain galactitol showing that unavoidable peeling reaction occurred under severe reaction conditions. Thus, oligosaccharide alditols were obtained from 50-h treatment of B-JGP and used for structural analysis.

Isolation and Purification of the Acidic Glycan Chains Present in B-JGP

Anion-exchange chromatography on DEAE-Sephadex A-25 of the oligosaccharide alditols obtained from B-JGP showed a carbohydrate-containing peak (A) eluted at about 0.08 M NaCl (Fig. 1.3). When the acidic fraction (A) was chromatographed on Sephadex G-25, two fractions, 1 and 2, were separated (not shown). These were analyzed for their sugar composition, and examined by TLC. Fraction 1 eluted near the void volume was found devoid of GalNAcol and to stay at the origin on TLC, representing uncleaved glycopeptides under the reaction conditions used. Fraction 2 showed two spots on TLC. These two components were separated by chromatography on Iatrobeads as in Fig. 1.4 to give A-1 and A-2. Yields: 1.8 mg and 2.9 mg (as hexose) from 25 mg (as hexose) of B-JGP, respectively.

Isolation and Purification of the Neutral Glycan Chains Present in B-JGP

The neutral component not bound to the above DEAE-Sephadex A-25 column was applied to a column of Bio-Gel P-4 (not shown). Subfractions 1-3 were obtained. Fraction 1 did not contain GalNAcol and did not migrate from the origin on TLC, indicating that it was the residual glycopeptides resulting from incomplete reaction of B-JGP with OH^-/BH_4^-. Fraction 2 was further purified by silica gel TLC and the major component was designated as N-1. Fraction 3 was chromatographed twice on Sephadex G-25 to give a neutral oligosaccharide alditol (denoted N-2), which gave a single spot migrating in a position identical to that of asialo A-1 when assessed by TLC. The yield of N-1 and N-2 from 25 mg B-JGP (as hexose) was 1.2 and 1.6 mg (as hexose), respectively.

Table 1.1. Amino acid and carbohydrate compositions of B-JGP before and after alkaline borohydride treatment. Values are molar ratios relative to Tyr set equal to 2.0. α-ABA, α-aminobutyric acid; -, not detected; n.d., not determined.

	Before treatment	After treatment
Asx	3.3	2.0
Glx	4.0	3.2
Ser	20.0	6.7
Gly	2.1	1.8
His	0.9	1.4
Arg	1.3	1.0
Thr	38.6	21.0
Ala	5.4	18.1
Pro	9.6	10.0
Tyr	2.0	2.0
Val	8.1	7.1
Met	0.4	0.6
Ile	1.8	1.7
Leu	2.1	1.8
Cys/2	1.4	0.0
Phe	1.3	0.7
Lys	6.1	3.3
α-ABA	-	17.6
Fuc	35.9	n.d.
Gal	65.4	n.d.
GalNAc	58.0	25.8
GlcNAc	8.6	9.2
NeuAc	35.9	n.d.
GalNAcol	-	29.1

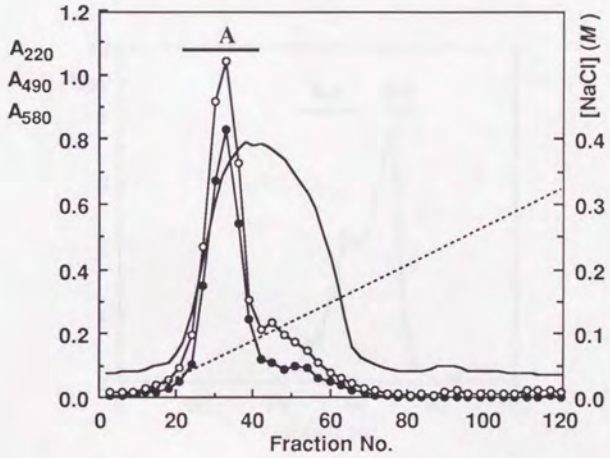


Fig. 1.3. DEAE-Sephadex A-25 anion-exchange chromatography of alkaline borohydride-treated B-JGP. B-JGP reacted with BH_4^-/OH^- for 50 h was charged on a DEAE-Sephadex A-25 column (1.5 x 40 cm, Cl^- form) and eluted with a linear gradient of NaCl in 10 mM Tris-HCl (pH 8.0) from 0 (500 ml) to 0.4 M (500 ml) (----). 5-ml fractions were collected and monitored by A_{220} (—), the phenol/sulfuric acid method (A_{490}, ○), and the resorcinol method (A_{580}, ●). The bar indicates fractions pooled to give A.

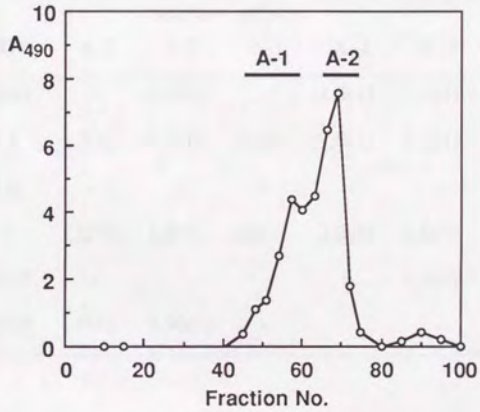


Fig. 1.4. Adsorption-partition chromatography of the acidic fraction (A) on Iatrobeads. The column (1.3 x 70 cm) was equilibrated and eluted with n-propyl alcohol/25 % aqueous ammonium/water = 6:1:1.5). 2-ml fractions were collected and analyzed for neutral sugar by the phenol/sulfuric acid method. Aliquots from fractions under the two peaks were examined by TLC (developed in n-propyl alcohol/25% aqueous ammonium/ water = 6:1:2.5), and the fractions containing a single component were pooled as indicated to give A-1 and A-2.

Structural Analysis of B-JGP Glycan Chains

(a) Carbohydrate Composition. Carbohydrate compositions of B-JGP and the oligosaccharide alditols therefrom are given in Table 1.2.

Table 1.2. Carbohydrate compositions of B-JGP and B-JGP-derived oligosaccharide alditols. The values for B-JGP and B-JGP-derived oligosaccharides are molar ratios relative to GalNAc and GalNAcol set equal to 1.0, respectively. The values in parentheses are the nearest integers.

	JGP	A-1	asialo-A-2	asialo-A-1	A-2	N-1	d-N-1	N-2
Fuc	0.63	-	0.90(1)	-	1.0(1)	1.0(1)	0.92(1)	0.77(1)
Gal	1.1	1.3(1)	1.3(1)	1.1(1)	1.0(1)	1.1(1)	1.0(1)	0.99(1)
GalNAc	1.0	-	-	-	-	-	-	-
GalNAcol	-	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)
GlcNAc	0.15	-	-	-	-	1.1(1)	-	-
NeuAc	0.62	1.0(1)	0.90(1)	-	-	-	-	-

(b) Carbohydrate Sequence. The results of methylation analysis of A-1, A-2, asialo A-1, asialo A-2, N-1, β-*N*-acetylhexosaminidase (β-HexNAc'ase)-treated N-1 (d-N-1), and N-2 (Table 1.3) gave the following information on their sequences as shown below.

(i) A-1 had an equimolar nonreducing terminal Gal and branched GalNAcol residues substituted both at *O*-3 and *O*-6. After sialidase treatment of A-1 the 3-*O*- substituted GalNAcol was newly formed at the expense of 3,6-di-*O*-substituted GalNAcol, indicating that sialic acid was linked α2→6 to the GalNAcol residue.

(ii) A-2 contained nonreducing terminal Fuc, 2-*O*-substituted Gal, and 3,6-di-*O*- substituted GalNAcol in a ratio of 1:1:1. The disappearance of the 3,6-di-*O*-substituted GalNAcol and appearance of 3-*O*-substituted GalNAcol on desialylation indicated that the sialic acid linkage in A-2 was the same as A-1.

(iii) N-1 had the terminal Fuc, 2-*O*-substituted Gal, terminal GlcNAc, and 3,6-di-*O*-substituted GalNAcol in an equimolar ratio. Removal of the GlcNAc residue by digestion of N-1 with Jack bean β-HexNAc'ase resulted in the conversion of 3,6-di-*O*-substituted GalNAcol into 3-*O*-substituted GalNAcol : GlcNAc was linked β1→6 to GalNAcol.

(iv) N-2 had one mole each of terminal Fuc, 2-*O*-substituted Gal, and 3-*O*-substituted GalNAcol.

Table 1.3. Partially methylated alditol acetates obtained in the methylation analysis of B-JGP-derived oligosaccharides. Molar ratios are given relative to the underlined values.

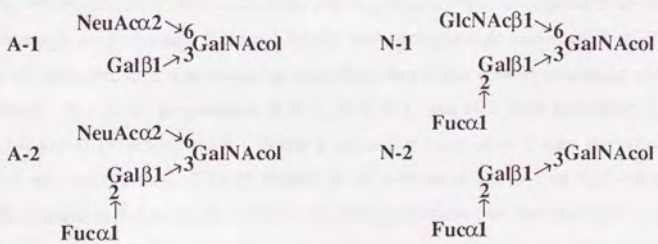
	A-1	A-2	asialo-A-1	asialo-A-2	N-1	d-N-1	N-2
Terminal Fuc	-	1.1	-	0.38	1.0	0.89	0.82
Terminal Gal	1.4	-	0.55	-	-	-	-
2-Substituted Gal	-	0.90	-	1.4	1.2	1.1	1.3
Terminal GlcNAc	-	-	-	-	0.94	-	-
3-Substituted GalNAcol	-	-	1.0	1.0	-	1.0	1.0
3,6-Substituted GalNAcol	1.0	1.0	-	-	1.0	-	-

(c) ^{1}H-NMR Spectroscopy. A summary of the assignments of the proton resonances for A-1, A-2, and N-1 is presented in Table 1.4. Chemical and spin-coupling constant data obtained from the 400 MHz ^{1}H-NMR spectra of these oligosaccharides were used to determine the anomeric form of their component monosaccharide residues as reported previously (Nasir-Ud-Din et al., 1986; Klein et al., 1988): Gal in A-1, A-2, and N-1, β; Fuc in A-2 and N-1, α; GlcNAc in N-1, β. The chemical shift values assignable to H-3_{ax} and H-3_{eq} of the NeuAc residues in A-1 and A-2 were consistent with those reported for α2→6-linked NeuAc (Nasir-Ud-Din et al., 1986).

Table 1.4. Chemical shifts for selected protons of B-JGP-derived oligosaccharides. Chemical shifts are measured in D_2O at 25°C and are expressed in ppm relative to methyl proton signal (0 ppm) of sodium 3-(trimethylsilyl)-propionate-2,2,3,3,-d_4.

Residue	Proton	A-1	A-2	N-1
GalNAcol	H-2	4.37	4.38	4.41
	H-3	4.05	4.08	4.09
	H-5	4.24	4.23	4.26
	NAc	2.04	2.03	2.08
Galβ1→3	H-1	4.47	4.58	4.58
Fucα1→2	H-1		5.26	5.23
	H-5		4.26	4.29
	CH_3		1.23	1.25
NeuAcα2→6	H-3_{ax}	1.69	1.69	
	H-3_{eq}	2.72	2.73	
	NAc	2.03	2.03	
GlcNAcβ1→6	H-1			4.56
	NAc			2.07

Based on these results, the structures of B-JGP oligosaccharides were deduced as:



DISCUSSION

A major glycoprotein (JGP) isolated and purified from the egg jelly of Japanese toad (*Bufo japonicus*). The structural features of B-JGP were the high content of carbohydrates (about 80% of the weight of B-JGP) and the high hydroxyamino acid content (Thr + Ser, 55 % of the total amino acid residues). More than 70% of the carbohydrate units in B-JGP were released by 110-h treatment with 0.1 N NaOH/1 M $NaBH_4$ at 37°C. Amino acid and carbohydrate analyses showed that these were linked through GalNAc to the hydroxyl groups of Thr and Ser residues. B-JGP isolated by the procedure described in this chapter accounted for 46% of the dry weight of the dialyzed egg jelly. This value may represent the minimum value because of the difficulty of quantitative recovery during purification.

When our analytical data for B-JGP were compared with those reported in the earlier studies of Kawai and Anno (1975) on the glycoprotein fraction obtained by pronase digestion of the *Bufo vulgaris* egg jelly, great similarities were shown in the relative carbohydrate and amino acid contents, the carbohydrate compositions (Gal, Fuc, GalNAc, GlcNAc, and NeuAc), a large proportion (more than 50%) of Ser and Thr, and in the fact that the next predominant amino acids were Pro, Ala, Val, and Lys. B-JGP was polydisperse, a molecular mass ranging from 100,000 Da to more than 4,000,000 Da. In spite of such high degree of polydispersity of B-JGP in molecular size, both amino acid and carbohydrate compositions of all fractions were practically the same. These results and the fact that the glycoprotein fraction obtained by Kawai and Anno had molecular mass of 100,000-150,000 Da, strongly suggested that B-JGP contained a regularly repeating sequence of 100-150 kDa glycoprotein units.

The studies reported here established that four major types of oligosaccharide units are linked through *O*-glycosidic linkages to the core polypeptide chain of B-JGP, and we determined their structures after releasing them from the protein core by treatment with alkaline borohydride. The molar proportions of A-1, A-2, N-1, and N-2 were estimated to be 0.34, 0.34, 0.14, and 0.19, respectively. While a precursor form of A-2 was identified as N-2, asialo A-1 was not detected. Though almost all the content of GlcNAc in JGP was accounted for by the amount of N-1 isolated, a minute amount of mannose was detected upon composition analysis. The earlier studies on blood group activity of the egg jelly coat from three different toad species showed the presence of either or both of glycan moieties carrying A- and H-determinants, though no structural information was reported (Schenkel-Brunner and Kothbauer, 1976; Yamamoto et al., 1977). The present results support the occurrence of the

glycan chains exhibiting blood group H activity but no glycan units carrying A-determinants were identified in JGP of *Bufo japonicus*.

Since B-JGP accounted for no less than 46% of non-dialyzable component of *Bufo* jelly coat, this molecule may possibly determine the physicochemical properties of the jelly coat. I studied Ca^{2+}-binding property of purified B-JGP and will discuss the function of B-JGP at fertilization in chapter III.

CHAPTER II ISOLATION OF A MAJOR GLYCOPROTEIN IN *XENOPUS LAEVIS* EGG JELLY COAT AND STRUCTURAL ANALYSIS OF ITS GLYCAN CHAINS

MATERIALS AND METHODS

Isolation and Purification of the Major Glycoprotein Component of the Jelly Coat from the South African Clawed Frog (Xenopus laevis) Eggs

Sexually mature frogs (*Xenopus laevis*) were purchased from a dealer in Japan. Ovulation was hormonally induced and unfertilized eggs were obtained (Hedrick and Nishihara, 1991). The jelly was solubilized, separated from the eggs, phenol-treated, dialyzed, subjected to Whatman DE52 anion-exchange chromatography, as described in chapter I. The products were designated as X-JGP0, X-JGP1, X-JGP2, and X-JGP3. The most major fraction in jelly extract, X-JGP3 was subjected to Sephacryl S-500 gel filtration for further purification as described in chapter I.

Alkaline Borohydride Treatment of the Glycoprotein and Purification of the O-Linked Oligosaccharides

X-JGP3 was incubated in 0.1 M NaOH/1 M $NaBH_4$ at 37°C for 48 h. Reaction mixture was neutralized with 1 M acetic acid and subjected to Bio-Gel P-6 chromatography (1.6 x 76 cm, equilibrated with 0.5% acetic acid). Glycopeptide fraction (I) and oligosaccharide fraction (II) were obtained. The oligosaccharide fraction (II) was desalted on a Sephadex G-25 column (1.3 x 103 cm, 5% ethanol) and fractionated by ion-exchange HPLC of Mono-Q into the neutral fraction (II-0) and two acidic fractions (II-1 and II-2) which were eluted with a 0-0.3 M NaCl gradient in 10 mM Tris-HCl (pH 8.0). Elution pattern was monitored by absorbance at 210 nm. Each fraction was desalted on a Sephadex G-25 column, and the purity was checked by TLC as described in chapter I. The II-1 fraction was subjected to preparative TLC as described in chapter I. To remove silica gel, each component thus obtained was applied to a DEAE-Sephadex A-25 column (0.6 x 3.5 cm, preequilibrated with 10 mM Tris-HCl, pH 8.0), eluted with 0.3 M NaCl/10 mM Tris-HCl (pH 8.0), and desalted on a Sephadex G-25 column (1.0 x 25 cm, 5% ethanol).

Chemical Analyses

The hexose content was estimated by the phenol/sulfuric acid method (Dubois et al.,

1956). Sulfate ion was identified and quantified by HPLC analysis of acid hydrolysate of a given sample. A sample (5-10 nmol) was hydrolyzed in 6 N HCl at 110 °C for 24 h and applied to TSKgel IC-anion PW column, which was equilibrated and eluted with 0.5 mM sodium phthalate in solution containing 0.036% boric acid, 0.05% sodium tetraborate, 0.2% (w/v) sodium gluconate, 12% acetonitrile, and 3% (v/v) 1-butanol at the flow rate of 1.2 ml/min. Elution was monitored and quantified by measuring the absorbance at 265 nm using sodium sulfate solution as standard. Amino acids and amino sugars were analyzed on a Waters PICO-TAG system after hydrolysis in 6 N HCl at 110°C for 24 h under N_2 and precolumn derivatization with phenylisothiocyanate (Heinrickson and Meredish, 1984). Animo acid analysis was done by Dr. Sadako Inoue (Showa University). Carbohydrate composition analysis was carried out as described in chapter I.

Desulfation by Mild Methanolysis

Oligosaccharides were methanolyzed in 0.5 ml of 0.05 N HCl/methanol at 25°C for 6 h. The methanolysates were evaporated to dryness and subjected to a DEAE-Sephadex A-25 column (Cl^- form, 0.6 x 3.5 cm, preequilibrated with 10 mM Tris-HCl, pH 8.0). Pass-through fractions were pooled and applied to a Sephadex G-10 column (1.0 x 25 cm, 5% ethanol). The fraction thus obtained was designated as the name of the fraction capped by MM-, e.g. MM-II-2 for II-2.

Methylation Analysis

Permethylation of oligosaccharides and the subsequent treatment were performed according to Anumula and Taylor (1992). GLC analysis of partially methylated alditol acetates was carried out on a Shimadzu GC-14A gas chromatograph with a capillary column (CBJ5, 30 m x 0.32 mm, Shimadzu) at 180-260°C at 2°C/min.

500 MHz ^{1}H-NMR Spectroscopy

Samples were processed as described in chapter I. ^{1}H-NMR spectra were recorded at 25°C with a Bruker AMX-500 NMR spectrometer. The chemical shifts were expressed in ppm relative to HDO proton signal set equal to 3.781 ppm. Measurements were made by Dr. Yutaka Muto and Dr. Satoru Watanabe (University of Tokyo).

FAB-MS Spectrometry

FAB-MS were measured by Dr. Anne Dell (Imperial College of Science, Technology and Medicine) with a VG Analytical ZAB-2SE FPD mass spectrometer fitted with a caesium ion gun operated at 30 kV and 20 kV in positive and negative modes, respectively. Data acquisition and processing were performed using VG Analytical Opus software. Sulfated oligosaccharides were deuteroacetylated or permethylated as described previously (Dell et al., 1994).

RESULTS

Isolation and Purification of Egg Jelly Glycoproteins from the Unfertilized Eggs of Xenopus laevis

The unfertilized eggs (160 ml) spawned by 20 females of *Xenopus laevis* were solubilized and phenol-treated. The aqueous phase was applied to a Whatman DE52 column and eluted with 0-1 M NaCl gradient in the presence of 2-mercaptoethanol. Four peak fractions of carbohydrate-containing material were obtained as shown in Fig. 2.1, and denoted as X-JGP0, X-JGP1, X-JGP2, and X-JGP3. The amino acid, carbohydrate, and sulfate compositions of these fractions are shown in Table 2.1. X-JGP3, which was eluted as the most abundant peak centered at ~0.4 M NaCl, was filtered on a Sephacryl S-500 column. The phenol-sulfuric acid-positive fractions were eluted in the molecular masses ranging from 4 x 10^6 to 1 x 10^6 (Fig. 2.2).

Isolation and Purification of the Glycan Chains Present in X-JGP3

The X-JGP3 sample (25 mg) was treated with 0.1 M NaOH/1 M $NaBH_4$ at 37°C for 48 h, and subjected to gel filtration on a Bio-Gel P-6 column (Fig. 2.3) after neutralizing with 1 M acetic acid. Two peak fractions positive for neutral sugar were eluted and denoted as I and II. Carbohydrate composition and TLC analyses showed that fraction I mainly contained glycopeptides and that almost all components of fraction II were released oligosaccharides (data not shown).

The oligosaccharide fraction (II) was applied to an anion-exchange HPLC on Mono-Q (Fig. 2.4), and three peaks were eluted at 0 M NaCl (II-0), ~0.1 M NaCl (II-1), and ~0.15 M NaCl (II-2), respectively. Fraction II-1 showed five spots on the TLC analysis. These five components were separated by preparative TLC, and designated II-1-1, II-1-2, II-1-3, II-1-4, and II-1-5. Fraction II-2 contained a single component.

Structural Determination of Acidic Glycan Chains Released from X-JGP3

Carbohydrate and sulfate composition of acidic glycan fractions was shown in Table 2.2. Of these six fractions, II-1-2, II-1-4, II-1-5, and II-2 were highly purified glycan fractions as judged from NMR and TLC analytical data, and subjected to structural analysis as shown below. The chemical shifts of these four fractions are summarized in Table 2.3.

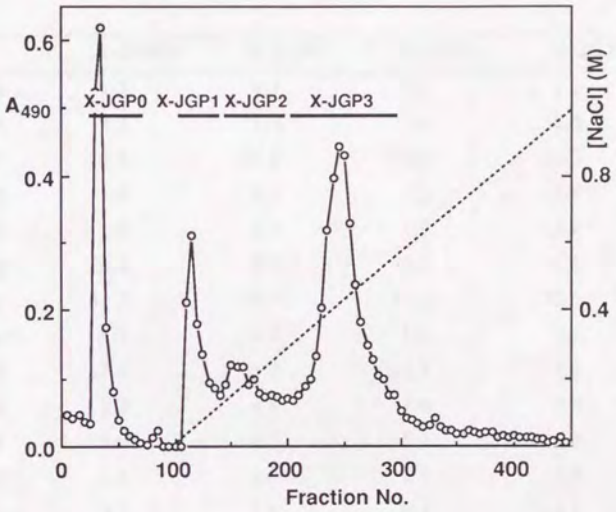


Fig. 2.1. Whatman DE52 anion-exchange chromatography of *Xenopus* egg jelly coat glycoprotein. Phenol-treated extract of *Xenopus* egg jelly was charged on a Whatman DE52 column (3.1 x 65 cm; Cl^- form) and eluted with a linear gradient of NaCl in 10 mM 2-mercaptoethanol/10 mM Tris-HCl (pH 8.0) from 0 to 1.0 M (----). 15-ml fractions were collected and analyzed for neutral sugar by the phenol/sulfuric acid method (A_{490}, ○). Fractions were pooled as indicated by the bars and designated as X-JGP0, X-JGP1, X-JGP2, and X-JGP3.

Table 2.1. Amino acid, carbohydrate, and sulfate compositions of X-JGPs. Values are molar ratios relative to total amino acid set equal to 100. Values in parenthesis are molar ratios relative to GalNAc set equal to 1.0.

	X-JGP0		X-JGP1		X-JGP2		X-JGP3	
Asx	1.7		4.0		1.2		2.2	
Glx	4.2		7.0		1.4		2.5	
Ser	19.3		21.8		24.0		18.2	
Gly	1.6		8.5		1.5		2.8	
His	1.6		1.7		1.9		2.4	
Arg	2.4		1.9		2.3		6.1	
Thr	41.3		24.7		41.2		32.5	
Ala	5.1		4.7		5.4		5.2	
Pro	7.6		5.4		6.7		7.1	
Tyr	1.2		4.4		1.6		0.8	
Val	5.0		6.2		5.4		6.6	
Met	1.4		1.0		1.1		1.8	
Ile	0.7		1.1		0.4		1.1	
Leu	1.5		2.2		0.7		2.1	
Phe	0.8		1.1		0.6		0.8	
Lys	4.5		4.2		4.6		7.8	
Fuc	38.1	(0.86)	47.5	(1.2)	71.6	(0.93)	71.4	(0.81)
Gal	66.5	(1.5)	83.2	(2.1)	131	(1.7)	123	(1.4)
GalNAc	44.3	(1.0)	39.6	(1.0)	77.0	(1.0)	88.1	(1.0)
GlcNAc	75.3	(1.7)	55.4	(1.4)	116	(1.5)	141	(1.6)
Sia	0.0	(0.0)	5.5	(0.14)	3.1	(0.04)	0.0	(0.0)
Sulfate	0.0	(0.0)	5.0	(0.13)	9.3	(0.12)	68.0	(0.77)

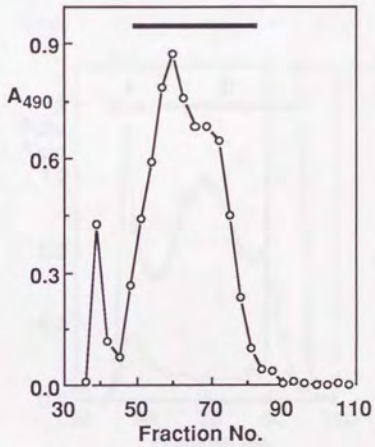


Fig. 2.2. Gel chromatography on a Sephacryl S-500 column of X-JGP3 obtained from the unfertilized egg jelly coat of *Xenopus laevis*. The column (2.8 x 156 cm) was equilibrated and eluted with 10 mM 2-mercaptoethanol/0.1 M NaCl/10 mM Tris-HCl (pH 8.0). Elution was monitored as described in the legend to Fig. 2.1. Fractions were collected every 9 ml, and pooled as indicated by the bar. V_0 and V_t are the void and total column volumes, respectively.

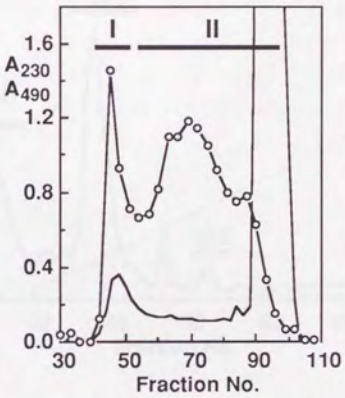


Fig. 2.3. Bio-Gel P-6 gel filtration of alkaline borohydride-treated X-JGP3. X-JGP3 treated with BH_4^-/OH^- for 48 h was applied to a Bio-Gel P-6 column (1.6 x 70 cm, equilibrated and eluted with 0.5% acetic acid). 1.5-ml fractions were collected and monitored by A_{230} (—) and the phenol/sulfuric acid method (A_{490}, ○). The bars indicate fractions pooled to give I and II.

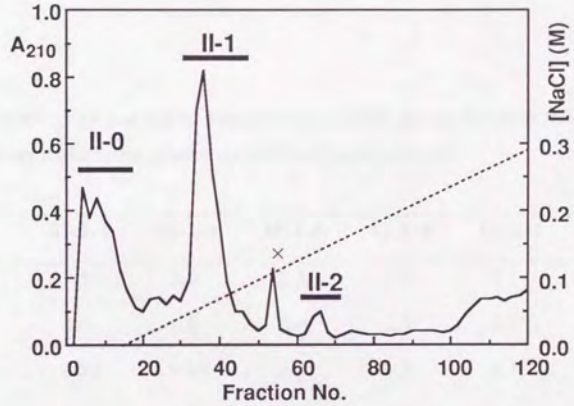


Fig. 2.4. Mono-Q anion-exchange chromatography of the oligosaccharide fraction (II) derived from X-JGP3. The fraction II in Bio-Gel P-6 chromatogram of Fig. 2.3 was applied to a Mono-Q HPLC and eluted with a linear gradient of NaCl in 10 mM Tris-HCl (pH 8.0) from 0 to 0.3 M (----). 0.5-ml fractions were collected and monitored by A_{210} (—). Fractions were pooled as indicated by the bars and designated as II-0, II-1, and II-2. The peak marked by × contained no carbohydrate.

Table 2.2. Carbohydrate and sulfate composition of acidic glycan fractions released from X-JGP3. Values are molar ratios relative to GalNAcol set equal to 1.0.

	II-1-1	II-1-2	II-1-3	II-1-4	II-1-5	II-2
Fuc	1.5	3.0	2.2	2.3	1.1	0.96
Gal	3.0	1.6	2.4	1.7	2.1	2.0
GalNAc	0.86	0.85	1.0	1.0	1.1	-
GlcNAc	1.1	1.5	1.4	2.0	1.6	1.0
GalNAcol	1.0	1.0	1.0	1.0	1.0	1.0
Sulfate	2.0	2.2	2.1	2.4	2.3	3.4

Table 2.3. ^{1}H NMR chemical shifts of the sulfated oligosaccharides from X-JGP3. Superscripts at the name of a sugar indicate the positions of glycosidic linkage showing the pathway from the residue toward the GalNAcol residue. n.d., not determined.

	H-1	H-2	H-3	H-4	H-5	H-6	H-6'	CH_3/NAc
II-2								
Fucα^{3,6}	5.12	3.68	3.92	3.80	n.d.	-	-	1.18
Galβ^{3}	4.57	3.72	4.35	4.28	n.d.	n.d.	n.d.	-
Galβ^{4,6}	4.64	3.62	4.33	4.28	n.d.	n.d.	n.d.	-
GlcNAcβ^{6}	4.59	3.95	3.88	4.04	3.83	4.39	4.39	2.06
GalNAcol	3.80/ 3.76	4.40	4.08	3.45	4.26	3.95	3.66	2.06
II-1-2								
Fucα^{2,3}	5.38	3.80	3.80	3.80	n.d.	-	-	1.23
Fucα^{3,3,3,3}	5.05	3.72	3.85	3.81	n.d.	-	-	1.17
Fucα^{3,6}	5.12	3.68	3.91	3.80	n.d.	-	-	1.18
Galβ^{3}	4.71	3.92	4.02	4.21	n.d.	n.d.	n.d.	-
Galβ^{4,6}	4.64	3.62	4.33	4.28	n.d.	n.d.	n.d.	-
GlcNAcα^{3,3,3}	4.88	4.11	3.89	3.63	4.22	n.d.	n.d.	n.d.
GlcNAcβ^{6}	4.63	3.95	3.89	4.05	3.84	4.40	4.39	n.d.
GalNAcα^{3,3}	5.25	4.35	4.06	n.d.	n.d.	n.d.	n.d.	n.d.
GalNAcol	n.d.	4.33	4.11	3.75	4.28	n.d.	n.d.	n.d.
II-1-5								
Fucα^{2,3}	5.38	3.80	3.80	3.80	n.d.	-	-	1.23
Galβ^{3}	4.71	3.92	4.02	4.21	n.d.	n.d.	n.d.	-
Galβ^{4,6}	4.64	3.67	4.35	4.30	n.d.	n.d.	n.d.	-
GlcNAcα^{3,3,3}	4.93	3.92	3.87	3.55	4.15	3.75	3.73	2.08
GlcNAcβ^{6}	4.61	3.79	3.72	3.83	n.d.	4.45	4.32	n.d.
GalNAcα^{a3,3}	5.18	4.25	3.93	4.02	n.d.	n.d.	n.d.	n.d.
GalNAcα^{b3,3}	5.22	4.34	4.06	4.03	n.d.	n.d.	n.d.	n.d.
GalNAcol	n.d.	4.29	4.17	3.77	4.23	n.d.	n.d.	n.d.

Structural Analysis of II-2

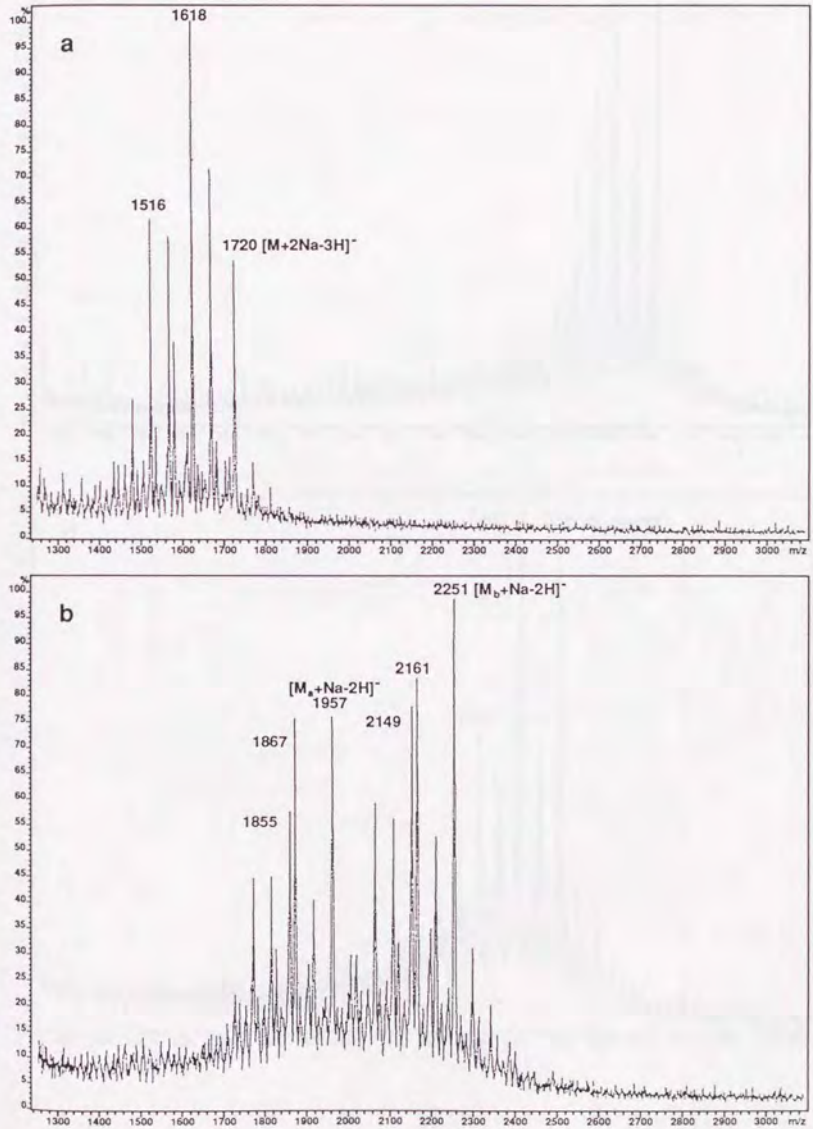
Composition and methylation analyses of II-2 and MM-II-2 (methanolysate of II-2) are summarized in Table 2.4. II-2 was trisulfated pentasaccharide-alditol with Fuc, Gal, GlcNAc, GalNAcol, and sulfate ion in the molar ratio of 1:2:1:1:3. FAB-MS of deuteroacetylated II-2 (Fig. 2.5a) showed a molecular ion $[M+2Na-3H]^-$ at m/z 1720. Composition analysis of MM-II-2 showed that II-2 was found to be completely desulfated on methanolysis and partial defucosylation also occurred. During methanolysis, two 3-*O*-substituted Gal residues disappeared with the concomitant increase of nonsubstituted Gal, showing that two galactose residues in II-2 were both sulfated on the 3-*O* position. Disappearance of 3,4,6-tri-*O*-substituted GlcNAc and the appearance of 3,4-di-*O*-substituted GlcNAc on the mild methanolysis of II-2 indicated 6-*O*-sulfation of GlcNAc in II-2. The detection of 4-substituted GlcNAc in MM-II-2 was presumably due to partial defucosylation and desulfation of 3,4,6-tri-*O*-substituted GlcNAc in II-2, which suggested GlcNAc residue was fucosylated at the 3-*O* position.

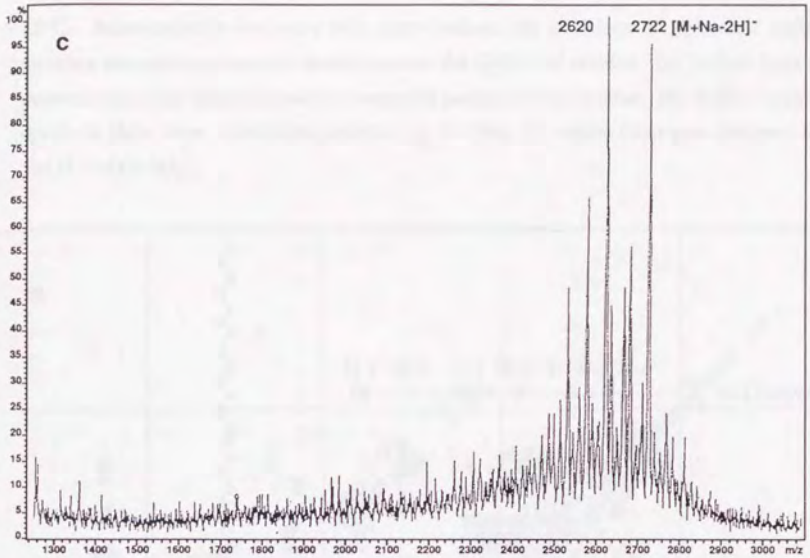
^{1}H-NMR spectra of II-2 are shown in Fig. 2.6. Resonances at 4.57 ppm and 4.64 ppm were assignable to H-1 of two Gal residues based on the previously reported data (Dabrowski, 1989) and spin-couplings of H2-H3 with an axial-axial orientation and H3-H4 with an axial-equatorial orientation which correspond to those of a galactopyranose in a normal chair form (Fig. 2.6b). Signals of H-1, H-3, and H-4 of the two galactose residues were significantly downfield-shifted, which was known to be typical of 3-*O*-sulfated Gal residues (Contreras et al., 1988; Capon et al., 1989; De Waard et al., 1991; Lo-Guidice et al., 1994). Those signals lined from 4.589 ppm (Fig. 2.6a) were assignable to proton resonance signals of β-GlcNAc residues, based on the previous data (Dabrowski, 1989), together with spin-coupling data indicating that H-1 through H-5 were all axial-oriented. In the GlcNAc residue, large downfield-shifts by at least 0.3 ppm were observed for H-6 and H-6' resonance signals, both of which resonated at δ=4.39 ppm. Downfield-shifts of H-6 and H-6' are characteristic of 6-*O*-sulfation (Strecker et al., 1989; De Waard et al., 1991; Lo-Guidice et al., 1994). Coincidence at δ=~4.38 ppm of H-6 and H-6' resonance signals in GlcNAc was a common feature to 3-fucosylated, 4-galactosylated, and 6-sulfated GlcNAc residues found in other glycan chains (Lo-Guidice et al., 1994; Brown et al., 1994). ^{1}H-NMR spectra also demonstrated the anomery of each sugar to be α for Fuc, β for both 2 Gal, and β for GlcNAc. Chemical shifts of H-2 (δ=4.40 ppm), H-4 (δ=3.45 ppm), and H-5 (δ=4.26 ppm) of GalNAcol were typical

Table 2.4. Composition and methylation analyses of II-2 and MM-II-2. Values for composition analysis are molar ratios relative to GalNAcol set equal to 1.0. Values for methylation analysis are molar ratios relative to 3,6-substituted GalNAcol set equal to 1.0.

	II-2	MM-II-2
Fuc	0.96	0.13
Gal	2.0	1.8
GlcNAc	1.0	1.1
GalNAcol	1.0	1.0
Sulfate	3.4	-
Terminal Fuc	0.26	0.22
Terminal Gal	-	1.1
3-Substituted Gal	0.29	-
4-Substituted GlcNAc	-	0.41
3,4-Substituted GlcNAc	-	0.10
3,4,6-Substituted GlcNAc	0.04	-
3,6-Substituted GalNAcol	1.0	1.0

Fig. 2.5. Negative-ion FAB-MS of deuteroacetylated oligosaccharides from X JGP3, (a) II-2, (b) II-1-5, (c) II-1-2, and (d) II-1-4. Sulfated oligosaccharides were deuteroacetylated and subjected to negative-mode FAB-MS measurement. Signals corresponding to molecular ions ($[M+2Na-3H]^-$ or $[M+Na-2H]^-$), underdeuteroacetylated ions, and losses of sodium sulfite from these ions were observed.




c
2620
2722 [M+Na-2H]⁻
%
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1300 1400 1500 1600 1700 1800 1900 2000 2100 2200 2300 2400 2500 2600 2700 2800 2900 3000 m/z

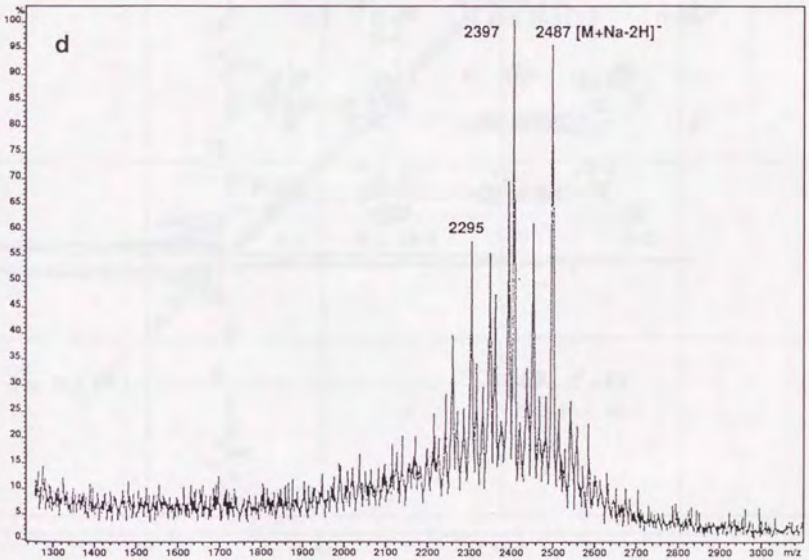
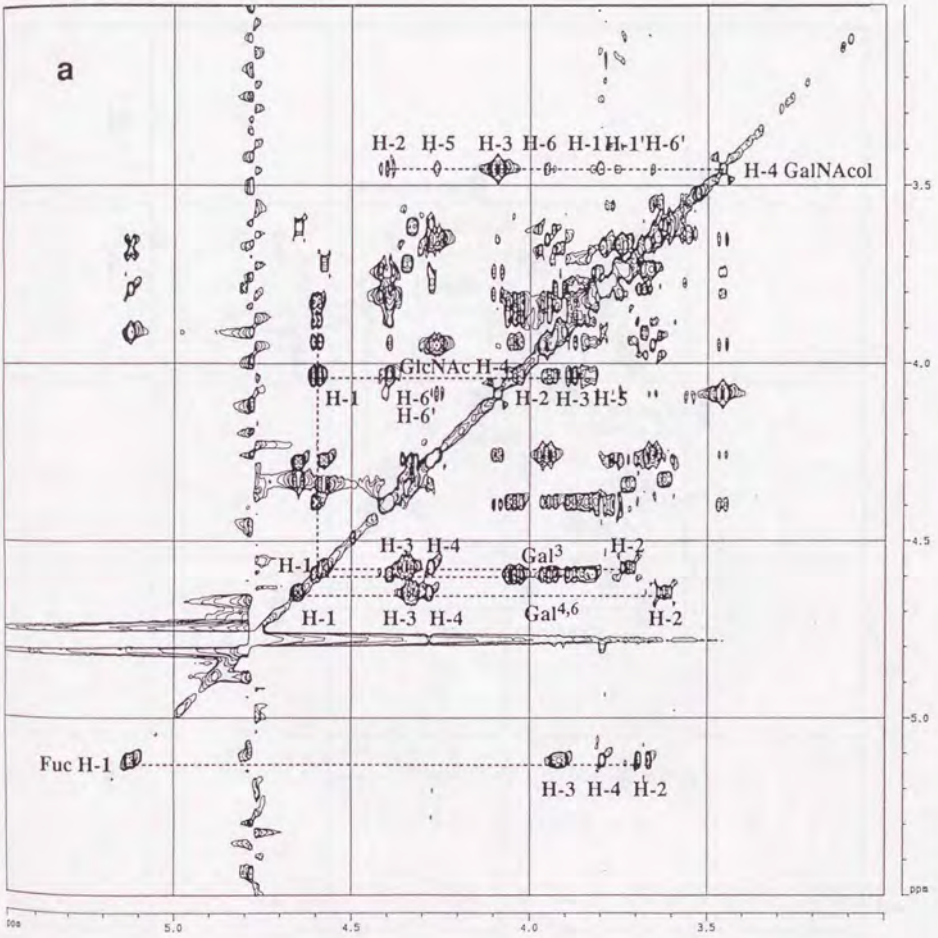
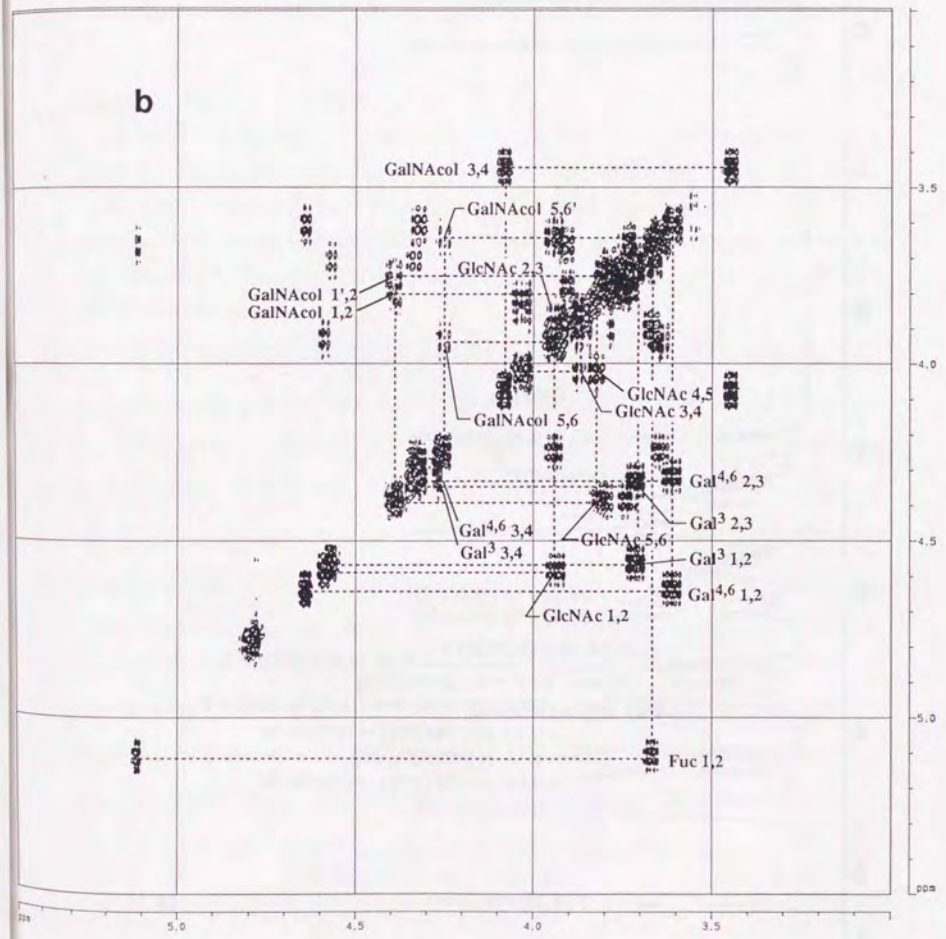

d
2397
2487 [M+Na-2H]⁻
2295
%
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
1300 1400 1500 1600 1700 1800 1900 2000 2100 2200 2300 2400 2500 2600 2700 2800 2900 3000 m/z

Fig. 2.6. 500 MHz (a) TOCSY, (b) COSY, and (c) 1D ^{1}H-NMR spectra of II-2 in D_2O at 25°C. Superscripts at the name of a sugar indicate the positions of glycosidic linkage showing the pathway from the residue toward the GalNAcol residue. (a) Broken lines are drawn to show the interconnection between the protons of one residue. (b) Broken lines are drawn to show some correlations present: e.g. GlcNAc 1,2 means cross peak between H-1 and H-2 of GlcNAc.

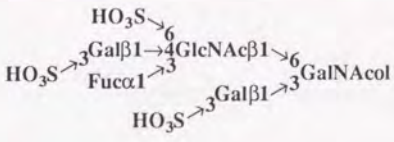



b
GalNAcol 3,4
GalNAcol 5,6'
GlcNAc 2,3
GalNAcol 1',2
GalNAcol 1,2
GlcNAc 4,5
GlcNAc 3,4
GalNAcol 5,6
Gal4,6 2,3
Gal3 2,3
Gal4,6 3,4
GlcNAc 5,6
Gal3 3,4
Gal3 1,2
Gal4,6 1,2
GlcNAc 1,2
Fuc 1,2
3.5
4.0
4.5
5.0
ppm
5.0
4.5
4.0
3.5

C
5.12 H-1 of Fuca
4.64 H-1 of Galβ1→4GlcNAcβ
4.59 H-1 of GlcNAcβ
4.57 H-1 of Galβ1→3GalNAcol
4.40 H-2 of GalNAcol
4.39 H-6 + H-6' of GlcNAcβ
4.33, 4.35 H-3 of Galβ
4.28 H-4 of Galβ × 2
4.26 H-5 of GalNAcol
4.08 H-3 of GalNAcol
4.04 H-4 of GlcNAcβ
GlcNAcβ1
3
6
3.45 H-4 of GalNAcol
Galβ1
3
1.18 CH3 of Fuca
ppm
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0

values for structure of core type 2, Galβ1→3(GlcNAcβ1→6)GalNAcol (van Halbeek et al., 1982a).

From all the above data, structure of II-2 was determined as:

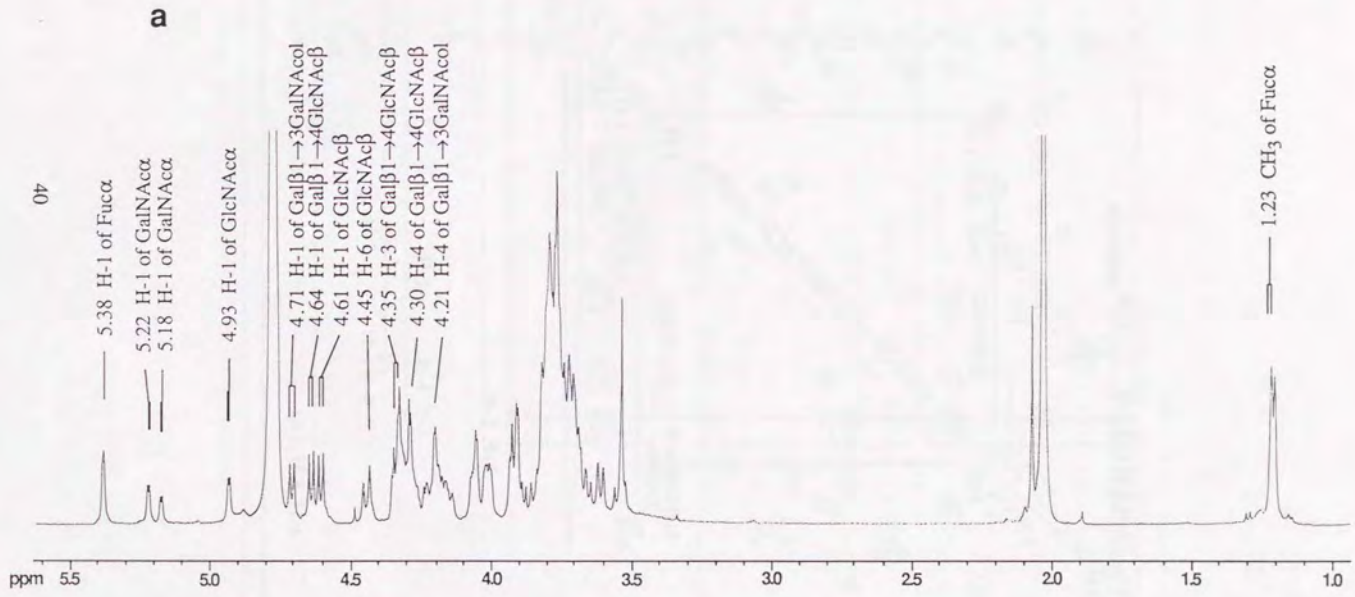


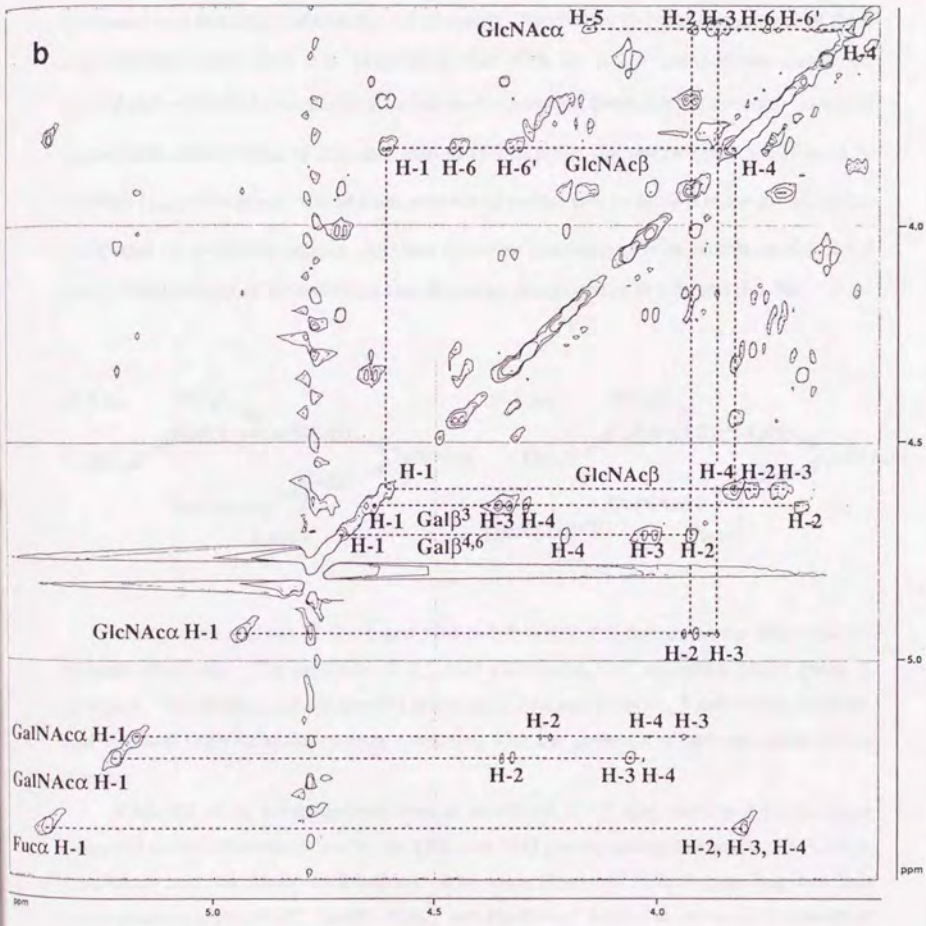
Structural Analysis of II-1-5

FAB-MS of deuteroacetylated II-1-5 (Fig. 2.5b) suggested that this fraction was a mixture of (Sulfate)$_2$Fuc$_1$Hex$_2$HexNAc$_2$HexNAcol (m/z 1957) and (Sulfate)$_2$Fuc$_1$Hex$_2$-HexNAc$_3$HexNAcol (m/z 2251). Combined with carbohydrate composition, II-1-5 was considered to consist of disulfated hexasaccharide-alditol and disulfated heptasaccharide-alditol with Fuc, Gal, GalNAc, GlcNAc, GalNAcol, and sulfate in the molar ratio of 1:2:1:1:1:2 and 1:2:1:2:1:2, respectively.

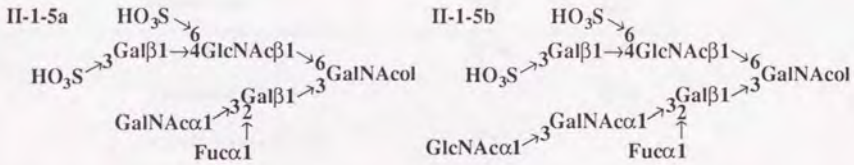
On 1D ^{1}H-NMR spectrum of II-1-5 (Fig. 2.7a), H-1 resonances of 4 α-glycoside and 3 β-glycoside residues were observed. The TOCSY spectrum (Fig. 2.7b) enabled us to assign these H-1 protons as indicated in Fig. 2.7a. The resonance signal intensities of H-1s of the 4 residues, α-Fuc (δ=5.38 ppm), β-Gal (δ=4.71 ppm), β-Gal (δ=4.64 ppm), and β-GlcNAc (δ=4.61 ppm), were identical and the signal intensities of α-GalNAc (δ=5.22 ppm), α-GalNAc (δ=5.18 ppm), and α-GlcNAc (δ=4.93 ppm) relative to those of the 4 residues were 0.6, 0.4, and 0.6, respectively. This suggested that these 4 residues of identical signal intensity constitute the common pentasaccharide structure together with GalNAcol residue. Chemical shift values of anomeric protons of three residues, α-Fuc (δ=5.38 ppm), α-GalNAc (δ=5.18 ppm), and β-Gal (δ=4.71 ppm), were completely corresponding to those of a partial structure of blood group A structure-containing oligosaccharide, GalNAcα1→3(Fucα1→2)Galβ1→3-(Galβ1→4GlcNAcβ1→6)GalNAcol (Dua et al., 1986). Since the relative signal intensity of α-GalNAc (δ=5.18 ppm) H-1 resonance was 0.4, 40% of II-1-5 were suggested to have this

Fig. 2.7. 500 MHz (a) 1D ^{1}H-NMR and (b) TOCSY spectra of II-1-5 in D_2O at 25°C. (b) Superscripts at the name of a sugar indicate the positions of glycosidic linkage showing the pathway from the residue toward the GalNAcol residue. Broken lines are drawn to show the interconnection between the protons of one residue.




b
GlcNAcα H-5
H-2 H-3 H-6 H-6'
H-4
H-1 H-6 H-6' GlcNAcβ
H-4
H-1
GlcNAcβ
H-4 H-2 H-3
H-1 Galβ3 H-3 H-4
H-2
H-1 Galβ4,6
H-4
H-3 H-2
GlcNAcα H-1
H-2 H-3
GalNAcα H-1
H-2
H-4 H-3
GalNAcα H-1
H-2
H-3 H-4
Fucα H-1
H-2, H-3, H-4
4.0
4.5
5.0
ppm
ppm
5.0
4.5
4.0

sequence. Based on the chemical shift values revealed by the TOCSY analysis, α-GlcNAc (δ=4.93 ppm) and α-GalNAc (δ=5.22 ppm) were, respectively, terminal α-GlcNAc (van Halbeek et al., 1982b) and 3-substituted GalNAc (the signal of H-3 was down-field shifted as compared with that of α-GalNAc (δ_{H-1}=5.18 ppm)). The relative H-1 signal intensities of these two residues were both 0.6, suggesting that 60% of II-1-5 components contained GlcNAcα1→3GalNAc sequence attached to the common pentasaccharide core. Unusual down-field shifted values of chemical shift of H-3 of β-Gal (δ_{H-1}=4.64 ppm) and H-6s of β-GlcNAc (δ_{H-1}=4.61 ppm) indicated the presence of sulfate groups on *O*-3 of the β-Gal residue and *O*-6 of the β-GlcNAc residue. All these data were consistent with the conclusion that II-1-5 was a 40:60-mixture of the following two structures, designated as II-1-5a and II-1-5b:



Methylation analysis of II-1-5 and MM-II-1-5 (Table 2.5) confirmed the above-shown linkage structures. The presence of 2,3-di-*O*-substituted Gal supported blood group A structure. The presence of comparable amounts of terminal GlcNAc, 3-substituted GalNAc, and terminal GalNAc residues were consistent with the presence of two structures shown above.

FAB-MS of the permethylated form of desulfated II-1-5 also confirmed the structure proposed above. Molecular ions at m/z 1402 and 1647 corresponding to $Fuc_1Hex_2HexNAc_2$-HexNAcol and $Fuc_1Hex_2HexNAc_3HexNAcol$ were observed. The A-type fragment ions corresponding to $HexNAc^+$, $HexHexNAc^+$, and $HexNAc_2^+$ were also observed in consistent with the proposed structure.

Table 2.5. Carbohydrate composition and methylation analyses of II-1-5 and MM-II-1-5. Values for composition analysis are molar ratios relative to GalNAcol set equal to 1.0. Values for methylation analysis are molar ratios relative to 3,6-substituted GalNAcol set equal to 1.0.

	II-1-5	MM-II-1-5
Fuc	1.1	0.90
Gal	2.1	2.0
GalNAc	1.1	1.0
GlcNAc	1.6	1.4
GalNAcol	1.0	1.0
Terminal Fuc	0.36	0.53
Terminal Gal	-	0.46
3-Substituted Gal	0.45	-
2,3-Substituted Gal	0.40	0.54
Terminal GalNAc	0.30	0.38
3-Substituted GalNAc	0.25	0.32
Terminal GlcNAc	0.28	0.35
4-Substituted GlcNAc	-	0.29
3,4-Substituted GlcNAc	0.14	-
3,6-Substituted GalNAcol	1.0	1.0

Structural Analysis of II-1-2

Composition analysis showed II-1-2 was disulfated nonasaccharide-alditol with Fuc, Gal, GalNAc, GlcNAc, GalNAcol, and sulfate in the molar ratio of 3:2:1:2:1:2. FAB-MS of deuteroacetylated II-1-2 (Fig. 2.5c) confirmed the composition as indicated by molecular ion $[M+Na-2H]^-$ at m/z 2722.

1D ^{1}H-NMR spectrum showed the presence of H-1 resonance signals of 5 α-glycosides and 3 β-glycosides as indicated in Fig. 2.8a. Assignments of resonance signals to the protons of these sugar residues were carried out on the basis of TOCSY (Fig. 2.8b) data, and summarized in Table 2.4. Chemical shift values of protons of α-Fuc (δ_{H-1}=5.12 ppm), β-Gal (δ_{H-1}=4.64 ppm), and β-GlcNAc (δ_{H-1}=4.63 ppm) were all identical to those of II-2, suggesting that II-1-2 shared the trisaccharide structure linked to *O*-6 of GalNAcol with II-2, which included sulfate groups attached to *O*-3 of Gal and *O*-6 of GlcNAc residues. Proton chemical shifts of α-Fuc (δ_{H-1}=5.38 ppm), α-GalNAc (δ_{H-1}=5.25 ppm), α-GlcNAc (δ_{H-1}=4.88 ppm), and β-Gal (δ_{H-1}=4.71 ppm) residues were very similar to those of the blood group A structure-containing tetrasaccharide of II-1-5b, except that H-1 and H-2 of the α-GlcNAc residue resonated differently from those of the α-GlcNAc in II-1-5b. The observed difference was most likely due to the fact that the α-GlcNAc residue was substituted by α-Fuc (δ_{H-1}=5.05 ppm) at the *O*-3 position. The presence of α-GlcNAc bearing α-Fuc on the *O*-3 position in II-1-2 was also indicated by detection of 3-*O*-substituted GlcNAc on methylation analysis (Table 2.6). All other partially methylated alditol acetates derived from II-1-2 and MM-II-1-2 were consistent with the following structure:

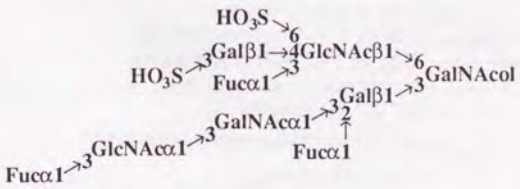
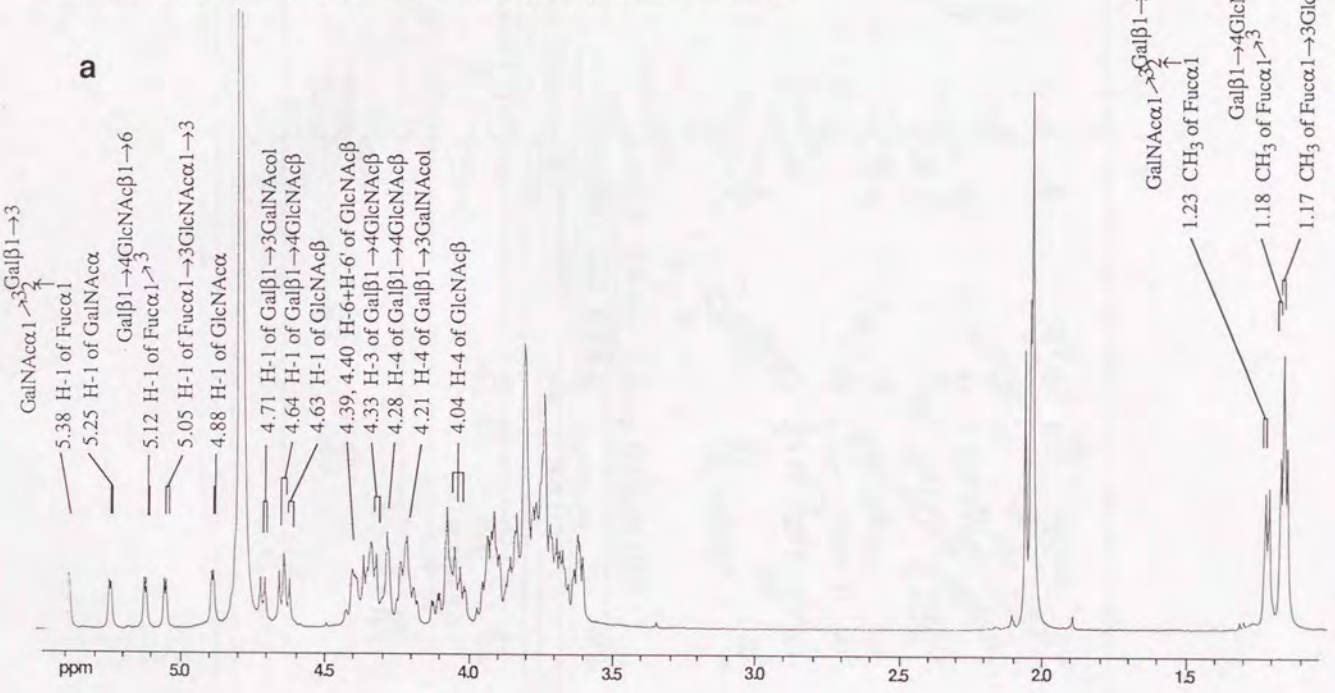


Fig. 2.8. 500 MHz (a) 1D ^{1}H-NMR and (b) TOCSY spectra of II-1-2 in D_2O at 25°C. (b) Superscripts at the name of a sugar indicate the positions of glycosidic linkage showing the pathway from the residue toward the GalNAcol residue. Broken lines are drawn to show the interconnection between the protons of one residue.



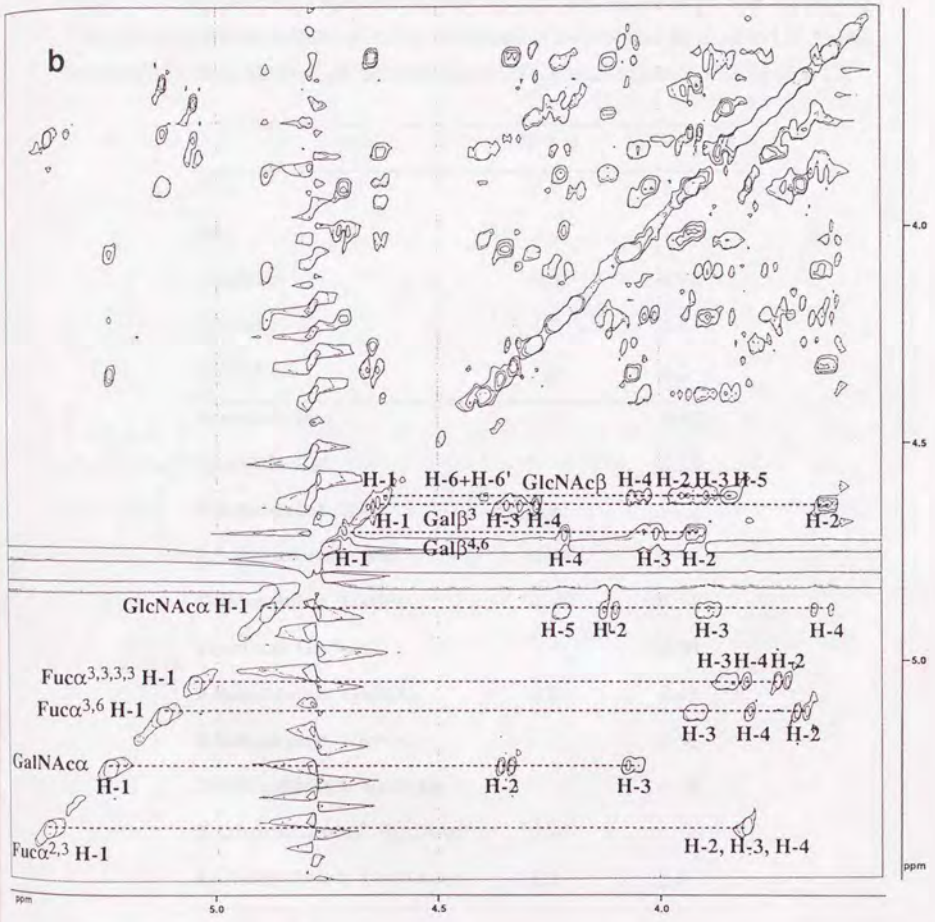

b
H-1 H-6+H-6' GlcNAcβ H-4 H-2 H-3 H-5
H-1 Galβ3 H-3 H-4
H-2
H-1 Galβ4,6
H-4
H-3 H-2
GlcNAcα H-1
H-5 H-2
H-3
H-4
H-3 H-4 H-2
Fucα3,3,3,3 H-1
Fucα3,6 H-1
H-3 H-4 H-2
GalNAcα
H-1
H-2
H-3
Fucα2,3 H-1
H-2, H-3, H-4
4.0
4.5
5.0
ppm
ppm
5.0
4.5
4.0

Table 2.6. Carbohydrate composition and methylation analyses of II-1-2 and MM-II-1-2. Values for composition analysis are molar ratios relative to GalNAcol set equal to 1.0. Values for methylation analysis are molar ratios relative to 3,6-substituted GalNAcol set equal to 1.0.

	II-1-2	MM-II-1-2
Fuc	3.0	1.6
Gal	1.6	1.8
GalNAc	0.85	0.92
GlcNAc	1.5	1.8
GalNAcol	1.0	1.0
Terminal Fuc	1.6	0.80
Terminal Gal	-	0.33
3-Substituted Gal	0.95	-
2,3-Substituted Gal	0.54	0.49
3-Substituted GalNAc	0.46	0.54
Terminal GlcNAc	-	0.30
3-Substituted GlcNAc	0.80	0.46
4-Substituted GlcNAc	-	0.36
3,4-Substituted GlcNAc	-	0.18
3,4,6-Substituted GlcNAc	0.08	-
3,6-Substituted GalNAcol	1.0	1.0

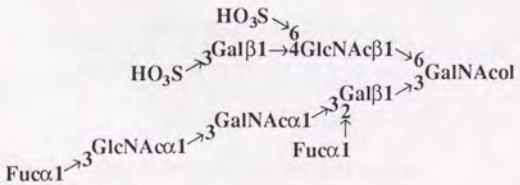
FAB-MS of permethylated derivative of desulfated II-1-2 confirmed the above structure. The molecular ion of the nonasaccharide was observed at m/z 1995, and those of its partially defucosylated forms were also observed at m/z 1821, and 1647 corresponding to $Fuc_2Hex_2HexNAc_3HexNAcol$ and $Fuc_1Hex_2HexNAc_3HexNAcol$, respectively. The partial defucosylation might have occurred under the solvolytic conditions used. The A-type fragment ions were also observed at m/z 434, 638, and 679 corresponding to $FucHexNAc^+$, $FucHexHexNAc^+$, and $FucHexNAc_2^+$, all consistent with the determined structure. $HexHexNAc^+$ (m/z 464) presumably arising from defucosylated material was also observed.

Structural Analysis of II-1-4

Composition analysis showed II-1-4 was disulfated octasaccharide-alditol with Fuc, Gal, GalNAc, GlcNAc, GalNAcol, and sulfate in the molar ratio of 2:2:1:2:1:2. FAB-MS of deuteroacetylated II-1-4 (Fig. 2.5d) confirmed the composition as indicated by ion $[M+Na-2H]^-$ at m/z 2487.

1D ^{1}H-NMR data of II-1-4 (Fig. 2.9) indicated the presence of 4 α- and 3 β-glycosides based on the H-1 proton resonances. By comparing with ^{1}H-NMR spectra of II-1-2 and II-1-5, all the H-1 protons were assigned as indicated in Fig. 2.9. Chemical shifts of the two H-1 protons, β-Gal (δ=4.64 ppm) and β-GlcNAc (δ=4.61 ppm), were corresponding to those of the disaccharide branch structure linked to *O*-6 of GalNAcol of II-1-5. The rest of H-1 protons, α-Fuc (δ=5.38 ppm), α-GalNAc (δ=5.24 ppm), α-Fuc (δ=5.05 ppm), α-GlcNAc (δ=4.88 ppm), and β-Gal (δ=4.71 ppm) were very similar to those of the pentasaccharide branch structure linked to *O*-3 of GalNAcol of II-1-2.

The structure of II-1-4 was thus deduced as:



Methylation analysis of II-1-4 and MM-II-1-4 (Table 2.7) confirmed the structure.

Fig. 2.9. 500 MHz ^{1}H-NMR spectrum of II-1-4 in D_2O at 25°C.

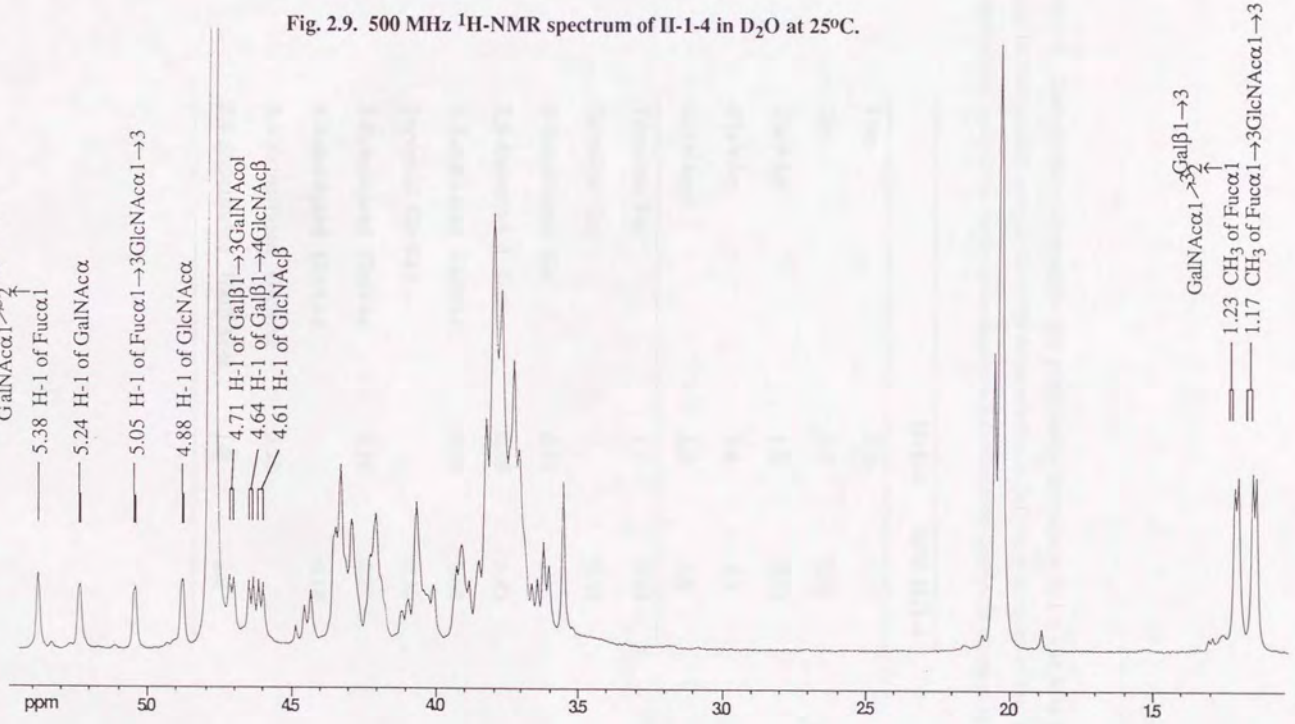


Table 2.7. Carbohydrate composition and methylation analyses of II-1-4 and MM-II-1-4. Values for composition analysis are molar ratios relative to GalNAcol set equal to 1.0. Values for methylation analysis are molar ratios relative to 3,6-substituted GalNAcol set equal to 1.0.

	II-1-4	MM-II-1-4
Fuc	2.3	1.3
Gal	1.7	2.0
GalNAc	1.0	0.92
GlcNAc	2.0	2.1
GalNAcol	1.0	1.0
Terminal Fuc	1.1	0.68
Terminal Gal	-	0.45
3-Substituted Gal	0.84	-
2,3-Substituted Gal	0.49	0.45
3-Substituted GalNAc	0.54	0.68
Terminal GlcNAc	-	0.30
3-Substituted GlcNAc	0.58	0.23
4-Substituted GlcNAc	-	0.18
3,4-Substituted GlcNAc	0.23	-
3,6-Substituted GalNAcol	1.0	1.0

Structure of the Major Glycan Chain in II-1-3

^{1}H-NMR spectrum (Fig. 2.10) showed that II-1-3 was a mixture of one major and two minor glycan chains. In the spectrum, six H-1 signals at δ=5.38, 5.17, 5.12, 4.71, 4.64, and 4.63 were observed and attributable to those of the major glycan chain. Shortage of the material prevented further detail analyses such as methylation analysis and FAB-MS measurement, but by comparison of the ^{1}H-NMR spectra with those of II-1-2 and II-1-5, structure of the major glycan chain in II-1-3 was deduced as:

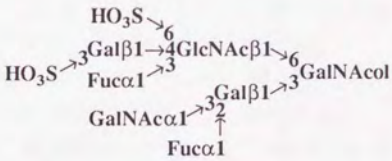
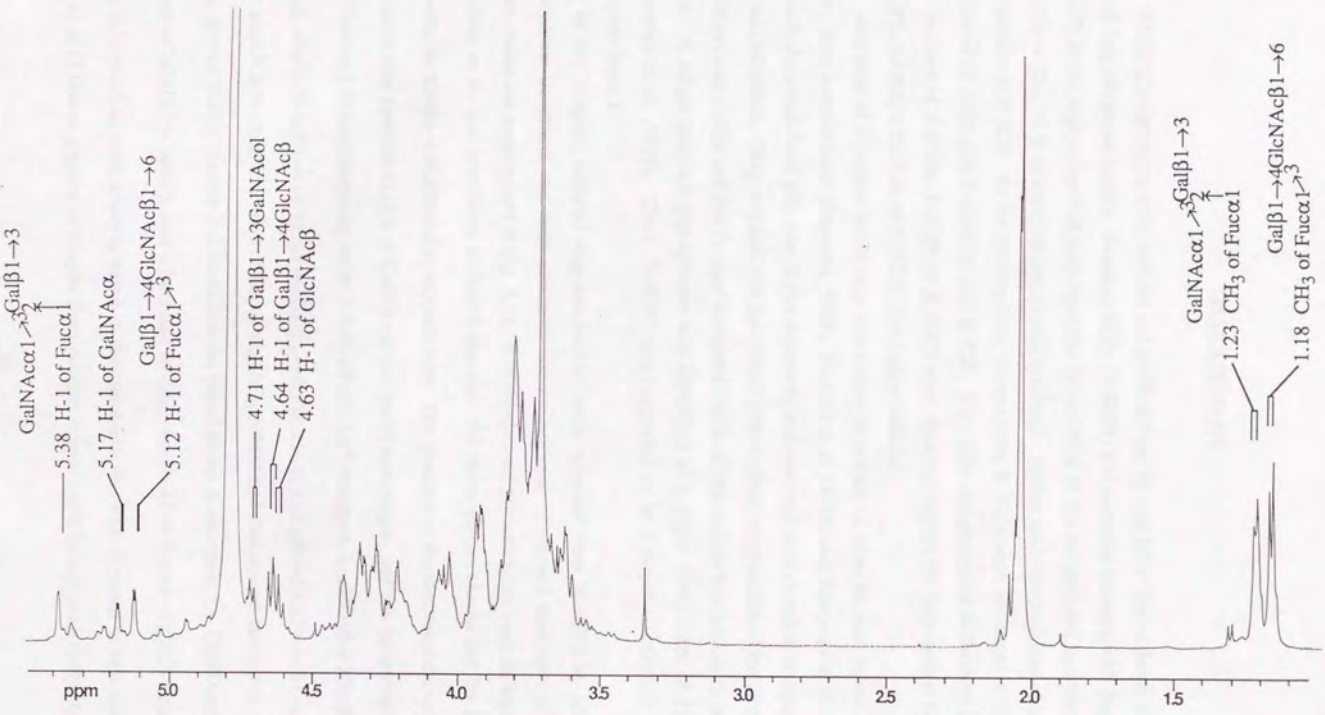


Fig. 2.10. 500 MHz ^{1}H-NMR spectrum of II-1-3 in D_2O at 25°C.



DISCUSSION

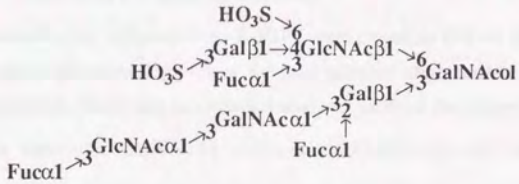
Major glycoproteins were isolated and purified from the egg jelly coat of South African clawed frog (*Xenopus laevis*). *Xenopus* JGPs (X-JGPs) had common features with *Bufo* JGP (B-JGP) in the high content of carbohydrates (about 80% of the weight) and hydroxyamino acid (Thr + Ser, 50 % of the total amino acid residues). Amino acid compositions of X.JPGs were similar to B-JGP. As for carbohydrate composition, X-JGPs were abundant in GlcNAc and devoid of sialic acid in contrast with B-JGP. Very little compositional differences among four fractions of X-JGPs, X-JGP0 to X-JGP3 were observed except for their sulfate content. X-JGP3, the major fraction of X-JGPs, was highly sulfated.

Jelly coat of *Xenopus laevis* eggs was known to consist of three distinct layers, J_1, J_2, and J_3, from inner to outer (Freeman, 1968). Hedrick et al. (1974) and Yurewicz et al. (1975) manually dissected these jelly coat layers separately and analyzed their chemical compositions after solubilization. They revealed that the overall carbohydrate composition of the three jelly coat layers was similar and that J_1 layer contained >90% of total sulfate ions present in the jelly layers. A single sulfated glycoprotein was identified as a major component of J_1 layer (Yurewicz et al. ,1975). Thus, X-JGP3 was suggested to be a major component of the innermost layer J_1.

In this chapter, sulfated oligosaccharides were released from X-JGP3 by alkaline borohydride treatment, and their structures were elucidated. Proposed structures of these glycan chains are summarized in Fig. 2.11. X-JGP3-derived glycan chains were all novel and identified as di- and trisulfated *O*-linked glycans. All these glycan chains had the core 2 structure, i.e. Galβ1→3(GlcNAcβ1→6)GalNAcol. The structure of the disulfated disaccharide or trisaccharide attached to *O*-6 of GalNAcol was novel and unique, and can be referred to as 3',6-disulfated *N*-acetyllactosamine or 3',6-disulfated Le^X structure, i.e. HO_3S→3Galβ1→4-(HO_3S→6)GlcNAcβ1→ or HO_3S→3Galβ1→4(Fucα1→3)(HO_3S→6)GlcNAcβ1→. The other branch arm attached to *O*-3 of the terminal GalNAcol had also unique structures. Other acidic glycan chains besides II-2 contained the blood group A structure, i.e. GalNAcα1→3-(Fucα1→2)Galβ1→, which was substituted by GlcNAcα1→3 or Fucα1→3GlcNAcα1→3. α-GlcNAc residues were scarcely found in glycoproteins, but were reported as non-reducing termini of *O*-linked glycans of mucins from porcine stomach cell linings (van Halbeek et al.,

Fig. 2.11. Proposed structures of sulfated glycan chains from X-JGP3.

II-1-2



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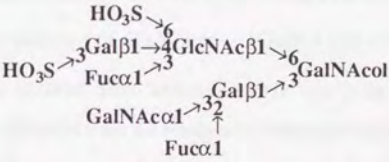

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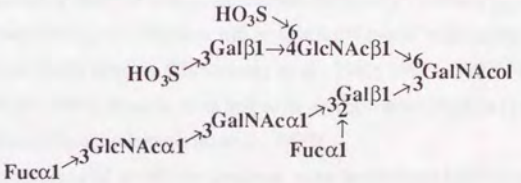
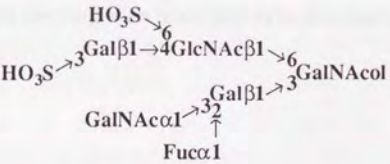


II-1-5a



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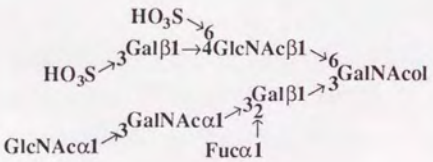
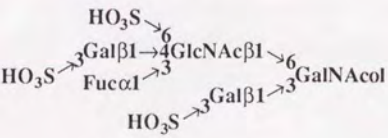


II-2



1982b) and of human foetal gastrointestinal mucins (Hounsell et al., 1989). To our knowledge, Fucα1→3GlcNAcα1→ structure was novel.

Neutral oligosaccharides released from X-JGP3 were eluted as II-0 on Mono-Q HPLC and found to constitute approximately 40% of the total released oligosaccharides as hexose. Carbohydrate composition, TLC, and methylation analyses showed the major neutral glycan chain to have the structure, GalNAcα1→3(Fucα1→2)Galβ1→3[Galβ1→4(Fucα1→3)-GlcNAcβ1→6]GalNAcol, which was a non-sulfated form of II-1-3 (data not shown). Strecker et al. (1995) reported the glycan structure of GlcNAcα1→3GalNAcα1→3(Fucα1→2)-Galβ1→3GalNAcol in materials derived from *Xenopus laevis* egg jelly coat, though oligosaccharides they studied were obtained from the whole jelly materials without purification as a glycoprotein.

Recently, a growing body of studies on sulfate-containing *O*-linked glycan chains has emerged although their biological functions still remain ambiguous: respiratory mucins from a patient suffering from cystic fibrosis (Mawhinney et al., 1987, 1992a, 1992b; Lamblin et al., 1991; Lo-Guidice et al., 1994), porcine zona pellucida glycoproteins (Hokke et al., 1994), and GlyCAM-1 as L-selectin ligand (Hemmerish et al., 1995).

Sulfated glycan chains of which the structure were determined here were shown to be important in calcium ion-binding and cortical granule lectin-binding as to be described in chapter III.

CHAPTER III CALCIUM- AND CORTICAL GRANULE LECTIN-BINDING PROPERTIES OF *BUFO JAPONICUS* AND *XENOPUS LAEVIS* EGG JELLY COAT GLYCOPROTEINS.

MATERIALS AND METHODS

Materials

B-JGP and X-JGP3 were purified from unfertilized egg jelly coats of *Bufo japonicus* and *Xenopus laevis* as described in chapter 1 and 2, respectively.

H-PSGP and L-PSGP were, respectively, purified from the unfertilized and fertilized eggs of rainbow trout by the methods previously reported (Inoue and Inoue, 1986; Inoue and Matsumura, 1979; Iwasaki et al., 1990; Shimamura et al., 1983). KDN-gp was isolated and purified from the ovarian fluid of rainbow trout by the procedure previously described (Kanamori et al., 1989).

Cortical granule lectin (CGL) from Xenopus laevis egg (Nishihara et al., 1986) and anti-CGL antibody (goat IgG) (Fabry and Hedrick, 1992) were provided by Dr. Song Yu and Dr. Jerry L. Hedrick (University of California, Davis). Peroxidase-conjugated rabbit anti-goat IgG was purchased from Cappel (Durham, NC).

Removal of Sialic Acid Residues from Sialoglycoproteins

The purified B-JGP (10 mg) was digested with *Arthrobacter ureafaciens* exosialidase (1.25 units, Nacalai, Kyoto) in 25 ml of 50 mM sodium acetate buffer (pH 5.5) at 37°C for 24 h. The digest was applied to chromatography on Sephacryl S-500 (column: 2.8 x 156 cm; equilibrated and eluted with 0.1 M NaCl/10 mM Tris-HCl, pH 8.0) and hexose-containing fractions were pooled and desalted by dialysis against water. The product thus obtained was designated as asialo-B-JGP.

Sialidase-treated H- and L-PSGP were prepared by removing the oligo/polysialyl chains from H- and L-PSGP (Kitajima et al., 1986, 1988): In brief, 30 mg of H-PSGP were treated with 4 ml of 50 mM acetic acid buffer (pH 4.0) at 37°C for 24 h to nonenzymatically remove the bulk of sialic acid residues (Kitazume et al., 1992). The hydrolysate was chromatographed on a column (2.3 x 160 cm, equilibrated and eluted with 0.1 M NaCl/5 mM Tris-HCl, pH 8.0) of Sephacryl S-200 to separate the partially desialylated H-PSGP from released free oligosialic acids. The partially desialylated H-PSGP was concentrated, dissolved in 4 ml of 50 mM acetic

acid buffer (pH 4.7), and digested thoroughly with *Arthrobacter ureafaciens* exosialidase (nacalai) by adding 0.125-0.5 unit at intervals of 5-6 h (total 1.25 units) and incubating at 37°C for 4 days. After the prolonged incubation, the digests were chromatographed on the same column of Sephacryl S-200 to separate them from free NeuGc released, and 9.8 mg of the sialidase-treated H-PSGP were obtained. Ten milligrams of sialidase-treated L-PSGP was prepared in a similar manner starting from 30 mg of L-PSGP.

Smith Degradation

The procedure was essentially the same as that of Spiro (1996). In brief, X-JGP3 (12 mg) was dissolved in 4 ml of 30 mM $NaIO_4$/50 mM sodium acetate buffer (pH 4.5) and kept in the dark at 4°C. After 48 h, 0.45 ml of 3% ethylene glycol was added to the reaction mixture to destroy excess periodate. After 30 min at 25°C, 85 mg of $NaBH_4$ and 3 ml of 0.5 M sodium borate buffer (pH 8.0) were added, and the reaction mixture was left overnight at 4°C. The solution was neutralized with 1 M acetic acid, and desalted by a Sephadex G-25 column. The eluate was evaporated to dryness and treated with 1.2 ml of 0.05 N HCl at 80°C for 1 h. The hydrolysate was neutralized with 0.05 N NaOH and subjected to gel filtration on a Sephadex G-50 column (1.3 x 110 cm, 0.1 M NaCl/10 mM Tris-HCl, pH 8.0). The product thus obtained was designated as SD-X-JGP3.

KDN-depleted KDN-gp prepared by the same Smith degradation procedure (Kanamori et al., 1990) was provided by Dr. Akiko Kanamori.

Mild Methanolysis

X-JGP3 (5 mg) was methanolyzed in 5 ml of 0.05 N HCl/methanol at 4°C for 48 h with agitating. The methanolysate was evaporated to dryness, dissolved in 1 ml of water, and subjected to chromatography on Sephadex G-50 column (1.3 x 110 cm, 0.1 M NaCl/10 mM Tris-HCl, pH 8.0). The product passed through the column and was designated as MM-X-JGP3.

Chemical Analyses

Carbohydrate composition analysis was carried out as described in chapter I. Sulfate ion was quantified as described in chapter II. Methylation analysis was carried out as described in chapter II.

Equilibrium Dialysis Method for Assaying of Calcium-Binding Activity of Glycoproteins

Washing and pre-treatment of dialysis tubing, the preparation of solutions, and other procedures followed essentially those reported by Potter et al. (1983) for determination of calcium binding with calmodulin. 1-ml aliquots of glycoprotein solution (1-40 mg/ml) were dialyzed first against 1 liter of 10 mM Tris-HCl, pH 7.3, containing 10 mM EDTA and then twice against 1 liter each of 10 mM Tris-HCl, pH 7.3. Subsequently, the solutions were dialyzed against 1 liter of 0.03-10 mM $CaCl_2$/37-148 kBq $^{45}CaCl_2$ (1.01 GBq/mg, NEN)-containing 10 mM Tris-HCl, pH 7.3 at 4°C for 48 h. After dialysis, solutions were taken from both sides of the dialysis membrane for measurement of radioactivity with a liquid scintillation counter and calcium ion concentrations were estimated from the radioactivity. Bound [Ca^{2+}] was calculated by subtraction of concentrations inside and outside the dialysis membrane. Glycoprotein concentrations inside the membrane were determined by measurements of the neutral sugar content by the phenol/sulfuric acid method (Dubois et al., 1956). All $^{45}Ca^{2+}$ experiments were carried out at the Radioisotope Center, University of Tokyo. For PSGP, sialidase-treated PSGP, KDN-gp, and KDN-depleted KDN-gp, 10 mM imidazole-HCl, pH 7.0 was used as the equilibration buffer.

ELISA Analysis for Cortical Granule Lectin-Binding to Glycoproteins

For lectin-binding assay, the solid-phase ELISA method was carried out as follows. Fifty µl of 0.1 mg/ml JGP in 0.15 M NaCl/10 mM Tris-HCl, pH 7.3 (TBS) were added to each well of a 96-well ELISA plate (NUNCLON, Nunc) and incubated at 37°C for 2h or at 4°C for 16 h. Nonspecific protein binding was blocked by incubating the plates with 1% BSA in TBS at 37°C for 2 h. Then, 50 µl of 0.1 mg/ml CGL in 1% BSA/TBS were added to each well and incubated at 25°C for 4 h. After rinsing the wells three times with TBS containing 0.05% Tween 20 (TBS-T), 50 µl of the anti-CGL antibody (25 µg/ml; diluted to 1:2500 with 1% BSA/TBS) were added and incubated at 37°C for 2 h. After rinsing the wells as described above, 50 µl of the secondary peroxidase-conjugated anti-goat IgG antibody, which was diluted to 1:1000 with 1% BSA/TBS, was added and incubated at 37°C for 1 h. After rinsing the wells as described above, the peroxidase substrate o-phenylene-diamine was added and incubated at 25°C for 30 min. The reaction was stopped by addition of 2 N H_2SO_4 and absorbance at 492 nm was measured on MTP-32 microplate reader (Corona Electric, Japan).

RESULTS

Binding of Ca^{2+} to JGPs

Binding of calcium ion to *Bufo* JGP and *Xenopus* JGP3 was studied by equilibrium dialysis using ^{45}Ca as tracer.

Fig. 3.1 shows the results of Ca^{2+} binding to *Bufo* JGP (B-JGP) in the presence of 1.3 mM Ca^{2+} that was corresponding to that in the De Boer ringer solution. The effect of B-JGP concentration on Ca^{2+} binding is shown in Fig. 3.1a. In this concentration range, the amount of the bound Ca^{2+} is a linear function of B-JGP concentration. This showed that B-JGP was capable of binding to Ca^{2+}. The Ca^{2+} binding capacity of B-JGP was lost when the sialic acid residues were removed by treatment with sialidase, indicating that the presence of the NeuAc residues is critically important for B-JGP to bind Ca^{2+}. When the bound Ca^{2+} (μmol) per B-JGP (mg) was plotted against B-JGP concentration, a rapid increase in the amount of the bound Ca^{2+} was observed at B-JGP concentration below 10 mg/ml (Fig. 3.1b).

The binding of Ca^{2+} to *Xenopus* JGP3 (X-JGP3) in the presence of 1.3 mM Ca^{2+} is shown in Fig. 3.2. As shown in Fig. 3.2a, the bound Ca^{2+} concentration is a linear function of X-JGP3 concentration in the concentration range used, showing that X-JGP3 also had Ca^{2+} binding activity like B-JGP. The amount of bound Ca^{2+} significantly decreased when the sulfate ions were partially removed from X-JGP3 by mild methanolysis, indicating that the sulfate ions are essential for X-JGP3 to bind Ca^{2+}. When the bound Ca^{2+} per X-JGP3 (μmol/mg protein) was plotted against X-JGP3 concentration, the bound Ca^{2+} rapidly increased at X-JGP3 concentration below 3 mg/ml (Fig. 3.2b).

The binding of Ca^{2+} to B-JGP was analyzed at two different concentrations, 3 mg/ml and 20 mg/ml (Fig. 3.3a), and the apparent binding constant (K_A) and the number of Ca^{2+} binding sites (n) obtained from the Scatchard plots are given in Table 3.1. A 1.5-fold increase in the n value was noted at the lower B-JGP concentration, whereas the K_A value showed a decrease to two thirds of that obtained at the higher B-JGP concentration. The binding of Ca^{2+} to X-JGP3 at 20 mg/ml was analyzed and plotted in Scatchard's manner in Fig. 3.3b, and the K_A and n obtained are given in Table 3.1. The binding constant of X-JGP3 to Ca^{2+} was 5 times as large as that of B-JGP.

Fig. 3.1. Effect of concentration of B-JGP on Ca^{2+} binding to B-JGP as studied by equilibrium dialysis. Calcium binding activity was measured by keeping [Ca^{2+}] constant at 1.3 mM and varying concentrations of B-JGP according to the procedure described in "Materials and Methods." (a) The change of concentration of the bound Ca^{2+} with B-JGP concentration and (b) the change of the bound Ca^{2+} in μmol per mg of B-JGP with B-JGP concentration are shown.

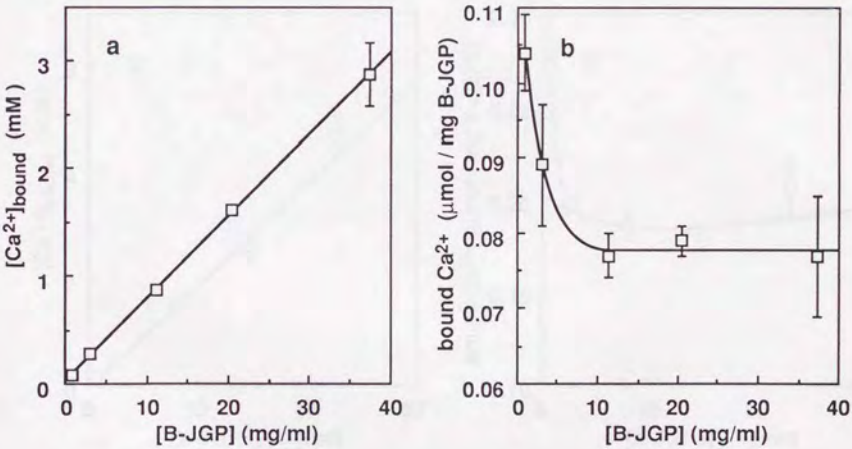


Fig. 3.2. Effect of concentration of X-JGP3 on Ca^{2+} binding to X-JGP3 studied by equilibrium dialysis. Calcium binding activity was measured by keeping [Ca^{2+}] constant at 1.3 mM and varying concentrations of X-JGP3 according to the procedure described in "Materials and Methods." (a) The change of concentration of the bound Ca^{2+} with X-JGP3 concentration and (b) the change of the bound Ca^{2+} in μmol per mg of X-JGP3 with X-JGP3 concentration are shown.

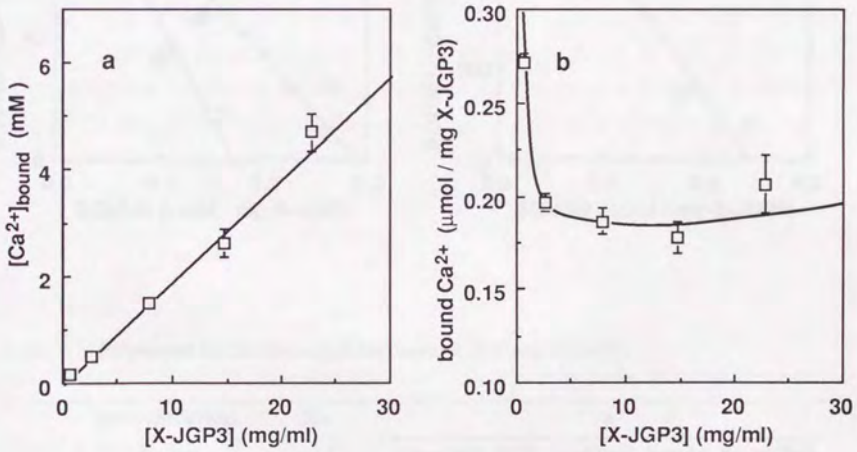


Fig. 3.3. Scatchard plots for calcium binding to (a) B-JGP and (b) X-JGP3 analyzed by equilibrium dialysis. In the case of B-JGP, the binding to Ca^{2+} was analyzed at two different concentrations at 3 mg/ml (■) and at 20 mg/ml (□). X-JGP3 binding to Ca^{2+} was analyzed at 20 mg/ml.

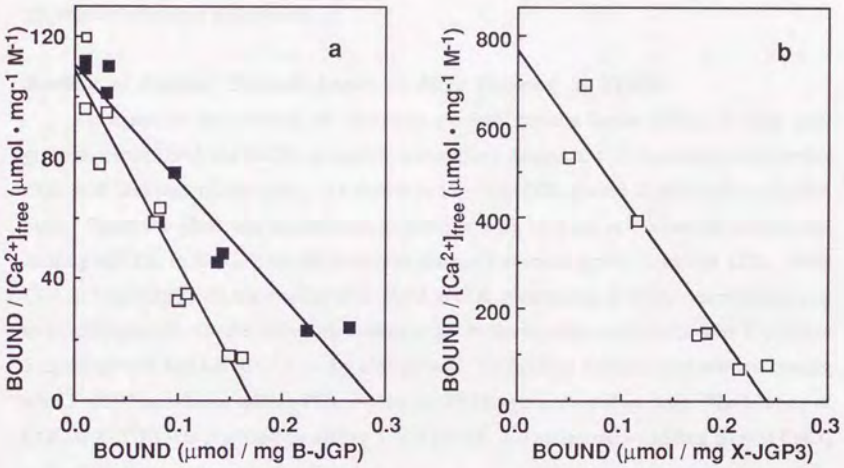


Table 3.1. Parameters for the binding of calcium to B-JGP and X-JGP3.

	concentration	K_A	n	
	mg/ml	M^{-1}	μmol/mg JGP	mol/mol NeuAc or sulfate
B-JGP	3	0.43×10^3	0.27	0.42
	20	0.63×10^3	0.17	0.26
X-JGP3	20	3.2×10^3	0.24	0.40

Binding of Calcium to Fish Oligo/Polysialic Acid-Containing Glycoproteins

Binding of Ca^{2+} to H-PSGP, L-PSGP, and KDN-gp was examined by the equilibrium dialysis, and the results are shown in Fig. 3.4 and Table 3.2. Binding constant for L-PSGP ($1.00 \times 10^3\ M^{-1}$) was one third of that for H-PSGP ($2.98 \times 10^3\ M^{-1}$), whereas the constants for H-PSGP and KDN-gp ($2.89 \times 10^3\ M^{-1}$) were essentially identical. These molecules lost their Ca^{2+} binding propensity when oligo/polysialyl chains were removed from PSGPs and KDN-gp, respectively (data not shown).

Binding of Cortical Granule Lectin to JGPs Detected by ELISA

To examine the binding of *Xenopus* cortical granule lectin (CGL) to jelly coat glycoproteins (JGPs), the ELISA procedure was applied using anti-CGL antibody for detecting CGL-JGP complex on the plate. As shown in Fig. 3.5, CGL bound X-JGP3 on the ELISA plate. When the plate was coated with X-JGP3 at 5 μg (1.3 μg as hexose)/50 μl/well, the binding of CGL to X-JGP3 on the plate was saturated above 2 μg/50 μl/well of CGL. With CGL at 5 μg/50 μl/well, the binding of X-JGP3 to CGL increased at X-JGP3 concentrations up to 5 μg/50 μl/well. On the following binding experiment, the plate was coated with X-JGP3 at 5 μg/50 μl/well and added CGL at 5 μg/50 μl/well. No binding was detected without coating with X-JGP3 or without adding CGL during the ELISA procedure (Fig. 3.6). The binding of CGL to X-JGP3 was inhibited by adding 5 mM EDTA, and recovered by adding 10 mM $CaCl_2$ to the EDTA containing solution (Fig. 3.6).

CGL-Binding to X-JGP3 and its Related Glycoproteins

CGL did not bind to other egg jelly glycoproteins, X-JGP0, 1, and 2, at the same JGP concentration (Fig. 3.7). As was already shown in Table 2.1, carbohydrate and amino acid compositions of X-JGP0-3 were almost identical. This showed that X-JGP3 was a specific ligand on the egg jelly of CGL since these four glycoproteins were able to account for 72% of glycoproteins present in the egg jelly. Methylation analysis of these glycoproteins showed the absence of 2,3-di-*O*-substituted Gal residues in X-JGP0, 1, and 2, while X-JGP3 contained Gal of the same substitution as the major structural element of *O*-linked glycan chain, Fucα1→2(GalNAcα1→3)Galβ1→.

Mild methanolysis and Smith degradation of X-JGP3 were carried out as described in "Materials and Methods," and the products were subjected to composition and methylation

Fig. 3.4. Scatchard plots for Ca^{2+} binding to fish oligo/polysialic acid-containing glycoproteins: (a) H-PSGP, (b) L-PSGP, and (c) KDN-gp.

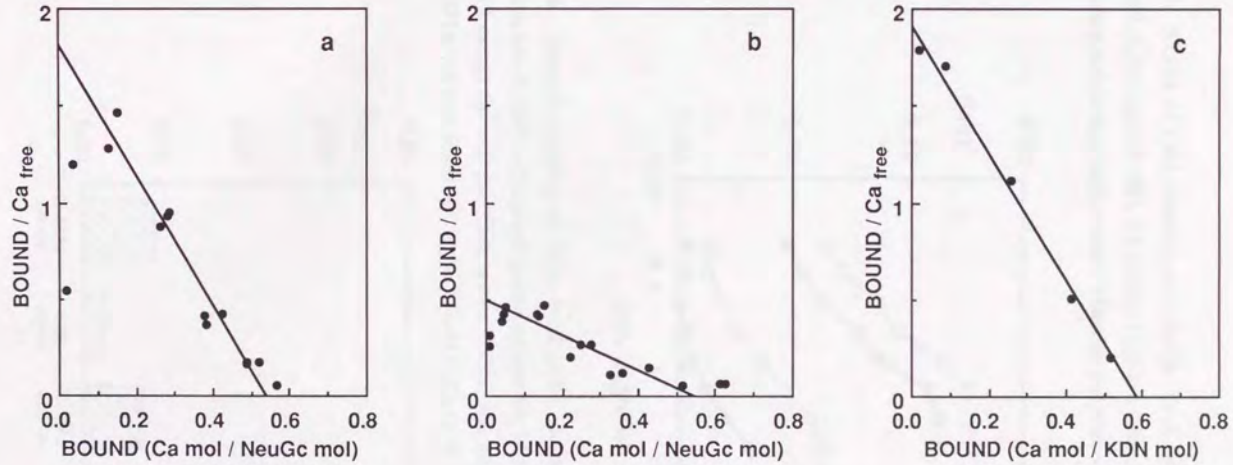


Table 3.2. Association constant and number of binding sites in the binding of Ca^{2+} to oligo/polysialic acid-containing glycoproteins.

	H-PSGP	L-PSGP	KDN-gp
K_A (M^{-1})	2.98×10^3	1.00×10^3	2.89×10^3
n (mol/mol Sia)	0.56	0.55	0.59

Fig. 3.5. ELISA of CGL binding to X-JGP3. Each well was coated with X-JGP3 at 50 μg/well (□), 5 μg/well (■), 0.5 μg/well (○), and 0.05 μg/well (●), and determined for CGL binding activity as described under "Materials and Methods."

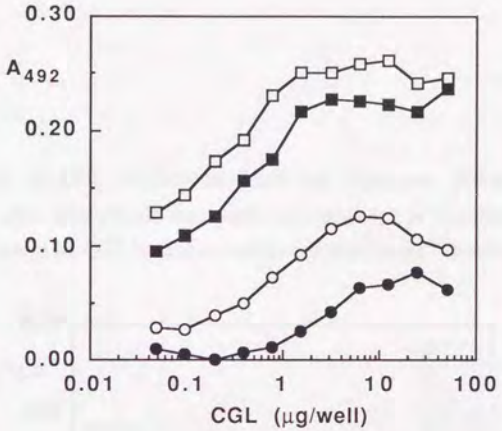


Fig. 3.6. Specific binding of CGL to X-JGP3 on the ELISA plate. Each well was coated with X-JGP3 at 5 μg/well except the well for "X-JGP3 minus," and added CGL at 5 μg/well except the well for "CGL minus." CGL was added with 5 mM EDTA to "X-JGP3+EDTA", or with 5 mM EDTA plus 10 mM $CaCl_2$ to "X-JGP3+EDTA+$CaCl_2$."

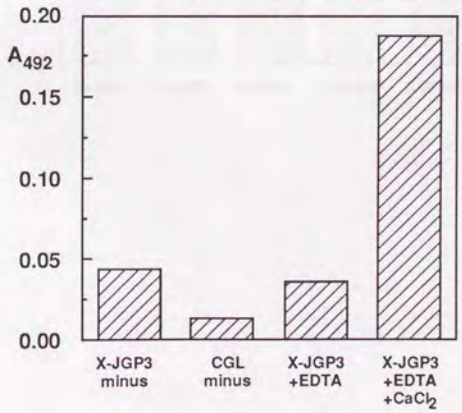
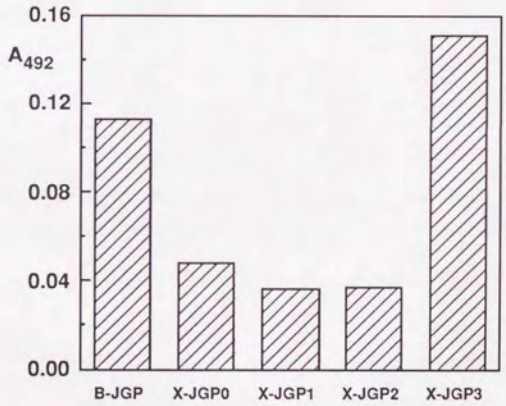


Fig. 3.7. ELISA of CGL binding to *Bufo* and *Xenopus* JGPs. Each well was coated with jelly coat glycoprotein from *Bufo japonicus* egg or *Xenopus laevis* egg at 5 μg/well, and determined for CGL binding activity as described under "Materials and Methods."

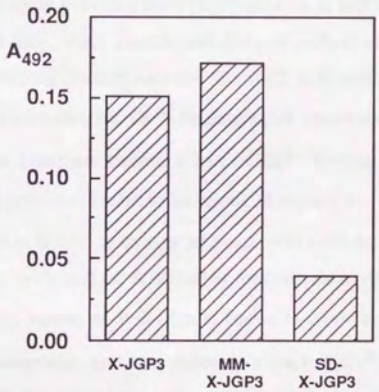


analyses (summarized in Table 3.3) and to ELISA for CGL-binding (Fig. 3.8). About half an amount of sulfate residues was removed from X-JGP3 by mild methanolysis. Little changes were observed in the CGL-binding to MM-X-JGP3 as compared with that to X-JGP3. On the other hand, CGL did not bind SD-X-JGP3 at all. Table 3.3 shows the absence of Fuc and 2,3-di-*O*-substituted Gal residues and the marked loss of non-substituted GalNAc residues on Smith degradation reaction. It was noted that SD-X-JGP3 was suggested not to have the 2,3-di-*O*-substituted Gal residue-containing structure as described above. B-JGP was also tested for CGL binding, and CGL was shown to bind to B-JGP to the same extent as X-JGP3 under the assay conditions used. The specificity of CGL binding is to be discussed in the "DISCUSSION" section.

Table 3.3. Carbohydrate and sulfate composition and methylation analyses of X-JGP and its derivatives, MM-X-JGP3 and SD-X-JGP3. The values for composition analysis are molar ratios relative to GalNAc set equal to 1.0. The values for methylation analysis are molar ratios relative to the sum of t-Gal, 3-Gal, and 2,3-Gal set equal to the value of Gal content in composition analysis.

	X-JGP3	MM-X-JGP3	SD-X-JGP3
Fuc	0.81	0.63	0.0
Gal	1.4	1.2	1.0
GalNAc	1.0	1.0	1.0
GlcNAc	1.6	1.7	1.5
Sulfate	0.77	0.45	0.77
t-Fuc	0.49	0.37	0.0
t-Gal	0.14	0.19	0.15
3-Gal	0.67	0.52	0.85
2,3-Gal	0.59	0.49	0.0
t-GalNAc	0.75	0.53	0.15
t-GlcNAc	0.16	0.14	0.20

Fig. 3.8. Determination of the property of CGL binding to X-JGP3 by ELISA. Each well was coated with X-JGP3 or its derivative at 1.27μg/well as hexose, and determined for CGL binding activity as described under "Materials and Methods."



DISCUSSION

Binding of Ca^{2+} to Egg Surface Glycoproteins

Since B-JGP accounted for no less than 46% of non-dialyzable component of *Bufo* egg jelly coat as described in chapter I, this molecule may possibly determine the physicochemical properties of the egg jelly coat, and consequently, play a critical functional role in fertilization. Ishihara et al. (1984) showed that the active components in Bufo egg jelly coat essential for fertilization were divalent cations, Ca^{2+} and/or Mg^{2+}. They showed that the dejellied eggs were fertilizable in a medium without any organic components of the egg jelly but containing 2-5 mM Ca^{2+} or Mg^{2+}, and concluded that the egg jelly coat plays a function of retaining Ca^{2+} and/or Mg^{2+} around the egg at the concentration level necessary for successful sperm entrance into the egg.

The study in this chapter provided both physicochemical and molecular basis for Ca^{2+}-binding property of Bufo jelly. First, equilibrium dialysis method showed that the purified B-JGP had a property of chelating calcium ions and that sialic acid residues in the oligosaccharide chains of B-JGP were responsible for Ca^{2+}-binding since removal of the sialic acid residues from B-JGP by sialidase treatment caused a loss of Ca^{2+}-binding property. Since B-JGP concentration in the egg jelly was found to be about 28 mg/ml as described in chapter I, the Ca^{2+} concentration bound to B-JGP in the egg jelly coat was estimated to be 2.3 mM, the value of which was consistent with that of fertilizable medium described above (i.e. 2-5 mM). Second, the Ca^{2+}-binding nature of B-JGP was found to change in biphasic manner as a function of B-JGP concentration, and two different modes of Ca^{2+}-binding were observed below and above the critical concentration of B-JGP, 7~10 mg/ml, when the data on calcium ion binding to B-JGP were plotted on Scatchard coordinates. In order to examine if the solution structure of B-JGP changes with B-JGP concentration, the viscosity of the B-JGP solution was determined by using a capillary viscometer as a preliminary experiment. An extensive concentration-dependent increase of viscosity was observed with B-JGP concentrations above ~7 mg/ml, whereas the increase of viscosity was less dependent on B-JGP concentrations below ~7 mg/ml (data not shown). Thus, the mode of Ca^{2+}-binding appeared to depend on the structure of B-JGP molecules in solution, although the exact

molecular mechanisms underlying JGP-Ca^{2+} interactions should be solved by other methods. It should also be noted that the toad eggs are known to undergo rapid swelling within several minutes after spawning in water. It was observed that the immersing of the unfertilized eggs in distilled water for 30 min caused an increase in volume of the jelly by about 3 times, and this resulted in the change in B-JGP concentration from about 28 mg/ml to less than 10 mg/ml.

Ishihara et al. (1984) have pointed out the significance of hydration of the jelly in pond water in Bufo fertilization. They showed that the jelly layers surrounding uterine eggs after oviposition contain cations at about 130 mM. They also suggested that during hydration process, Na^+ and K^+ may be released faster than Ca^{2+} and Mg^{2+}, so that the low salinity conditions required for the sperm motility and a certain level of the divalent cations essential for gamete fusion are both ensured. The present study showed the lower K_A and higher n values in Ca^{2+} binding under the low JGP concentration compared with the values obtained under high JGP concentration. The dissociation constant (K_D) of Ca^{2+}-binding to B-JGP under low B-JGP concentration was calculated to be 2.3×10^{-3} M, whereas this value was 1.6×10^{-3} M under the high JGP concentration. Thus 50% increment of both n and K_D values under the low JGP concentration as in the hydrated jelly favors in retaining Ca^{2+} and keeping its concentration just necessary for fertilization.

Fractionation of *Xenopus* egg jelly glycoproteins (JGPs) on anion-exchange chromatography revealed that JGPs were separable into 4 major glycoproteins: JGP0, JGP1, JGP2, and JGP3. These glycoproteins accounted for 72% of the total glycoprotenaceous components in the egg jelly, and they were found to have similar carbohydrate and amino acid compositions except for the content of sulfate or sialic acids. Among these JGPs, *Xenopus* JGP3 (X-JGP3) was a major sulfated component (approximately 50% as a neutral sugar) of the *Xenopus* egg jelly, and was also shown to bind Ca^{2+} via sulfate residues on glycan chains. Association constant of X-JGP3 to Ca^{2+} was 5 times as high as that of B-JGP, possibly due to preferential binding nature of sulfate ions in X-JGP3 to Ca^{2+} compared with the carboxylate ion of sialic acid in B-JGP. The change of Ca^{2+}-binding nature in biphasic manner was also observed in X-JGP3 as was the case with B-JGP, but the transition concentration of Ca^{2+} was below 3 mg/ml for X-JGP3, which was lower than that for B-JGP.

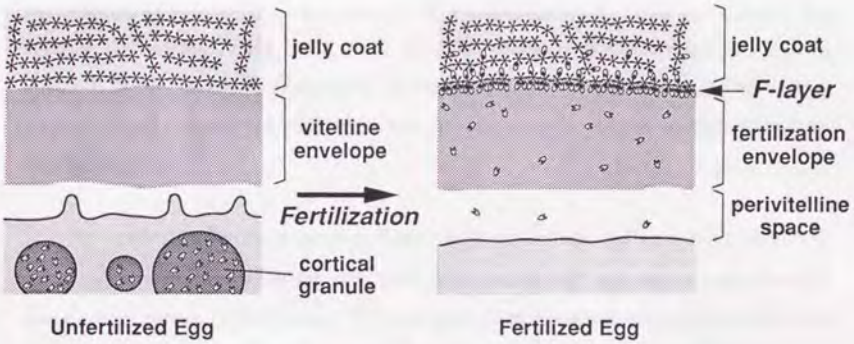
The Ca^{2+} binding nature of egg surface macromolecules would be essential for the eggs that are spawned into fresh water, because various cations are rapidly diffused out from the cell

surface into water immediately after the eggs go into outside. Like *Bufo* and *Xenopus*, rainbow trout put their eggs into fresh water at fertilization. Previous results showed that the eggs of rainbow trout were also surrounded by such polyanionic glycoproteins as H-PSGP, L-PSGP, and KDN-gp. They contain oligo/polysialic acid chains as acidic moieties. The Ca^{2+} binding property of those amphibian egg jelly coat glycoproteins prompted me to test their ability to bind to Ca^{2+}, and it was revealed that all of them shared calcium binding nature with association constants (KA) of 2.98×10^3 for H-PSGP, 1.00×10^3 for L-PSGP, and 2.89×10^3 M^{-1} for KDN-gp. Since the removal of oligo/poly(NeuGc) and oligo/poly(KDN) from PSGPs and KDN-gp abolished their Ca^{2+} binding capacities, the carboxylate groups of these macromolecules must have been critically involved in the binding of Ca^{2+}.

H-PSGP (molecular weight, about 200 K) is a characteristic constituent of cortical granule in the unfertilized eggs of rainbow trout (Inoue and Inoue, 1986; Inoue et al., 1987; Kitajima et al., 1988). The 200-kDa PSGP was shown to be made up of tandem repetitions of a glycotridecapeptide unit. In every repeating unit there are three attachment sites for oligo /polysialylglycan chains (Inoue and Inoue, 1986; Kitajima et al., 1986). Upon fertilization H-PSGP was found to undergo rapid proteolytic depolymerization into the repeating unit or L-PSGP (molecular weight, 9 K) (Inoue and Inoue, 1986). In the present study, the effect of molecular size of PSGP on calcium binding with PSGP was studied by comparison of H- and L-PSGPs which differ only in size. Ca^{2+} binding affinity for L-PSGP was 1/3 of that for H-PSGP, as studied by equilibrium dialysis, whereas the number of binding sites (n) did not change on going from H-PSGP to L-PSGP. These findings have implications for the mechanism of calcium release at fertilization. According to this view, the Ca^{2+} ions bound to H-PSGP represent a reservoir of calcium for liberation at fertilization (upon conversion of H-PSGP to L-PSGP). And L-PSGP is considered to function as a reservoir for Ca^{2+} in the perivitelline space surrounding an early embryo.

Binding of Cortical Granule Lectin to Jelly Coat Glycoproteins

At fertilization of *Xenopus laevis* egg, it was known that fertilization layer (F-layer) was formed between the vitelline envelope and the innermost jelly layer J_1 (Grey et al., 1974) (Scheme 3.1). The formation of F-layer was recognized as one of mechanisms of the block to polyspermy (Schmell et al., 1983). F-layer was formed by the interaction of a lectin released from cortical granule at fertilization and a ligand located between the vitelline envelope and the innermost jelly layer (Wyrick et al., 1974; Greve and Hedrick, 1978). Cortical granule lectin (CGL) was isolated (Nishihara et al., 1986) and ligand molecules were proposed (Birr and Hedrick, 1992; Yoshizaki, 1994). However, no structural information or molecular base of the lectin-ligand interaction was presented until now.



Scheme 3.1. At fertilization of *Xenopus laevis* egg, F-layer is to form between the vitelline envelope and the innermost jelly layer, assumably by interaction of CGL (Ⴤ) and glycan chains on X-JGP3 (*****).

X-JGP3 was expected to be a major glycoprotein of the innermost jelly layer J_1 based on the evidence that X-JGP3 was found to be a dominant sulfated glycoprotein in the egg jelly coat, and that only the innermost jelly layer J1 was previously shown to contain sulfate residues. Furthermore, since CGL was found to form lectin-ligand complex in the innermost jelly layer, X-JGP3 was strongly suggested to be the ligand for CGL. In this chapter, the interaction of CGL and X-JGP3 was examined by ELISA method.

CGL was shown to bind X-JGP3 at low Ca^{2+} concentration. CGL did not bind other X-JGP components (X-JGP0, 1, and 2), indicating that X-JGP3 was an endogenous ligand for CGL. Interestingly, B-JGP was found to act as a ligand for CGL. It was previously reported *Bufo japonicus* and *Xenopus laevis* egg jelly coats contained structurally related antigens which constitute CGL ligands (Hedrick and Katagiri, 1988). The present study gave a molecular basis for this observation.

The structures of *O*-linked glycans of B-JGP and X-JGP3, as was determined in chapter I and II, respectively, contained the common structure: Fucα1→2Galβ1→3(GlcNAcβ1→6)-GalNAc, and this structural element could constitute a determinant for binding of CGL. Methylation analysis showed X-JGPs other than X-JGP3 did not have this structural element as was already discussed in "RESULTS" section. MM-X-JGP3 bound CGL because it retained the structure as evidenced by the presence of 2,3-di-*O*-substituted Gal and terminal Fuc residues. On the other hand, SD-X-JGP3, which was completely defucosylated and lost the structure, did not bind CGL as expected. Accordingly, CGL is suggested to bind specifically Fucα1→2Galβ1→3GalNAc structure, where β1→6-linked GlcNAc to the GalNAc residue may be necessary.

In conclusion, by using purified JGPs I have shown that JGPs are the biologically functional components of the egg jelly coat. They retain Ca^{2+} and keep its concentration necessary for successful fertilization. They are also natural ligands of cortical granule lectin to play a role in polyspermy block by forming the barrier structure around the fertilized eggs.

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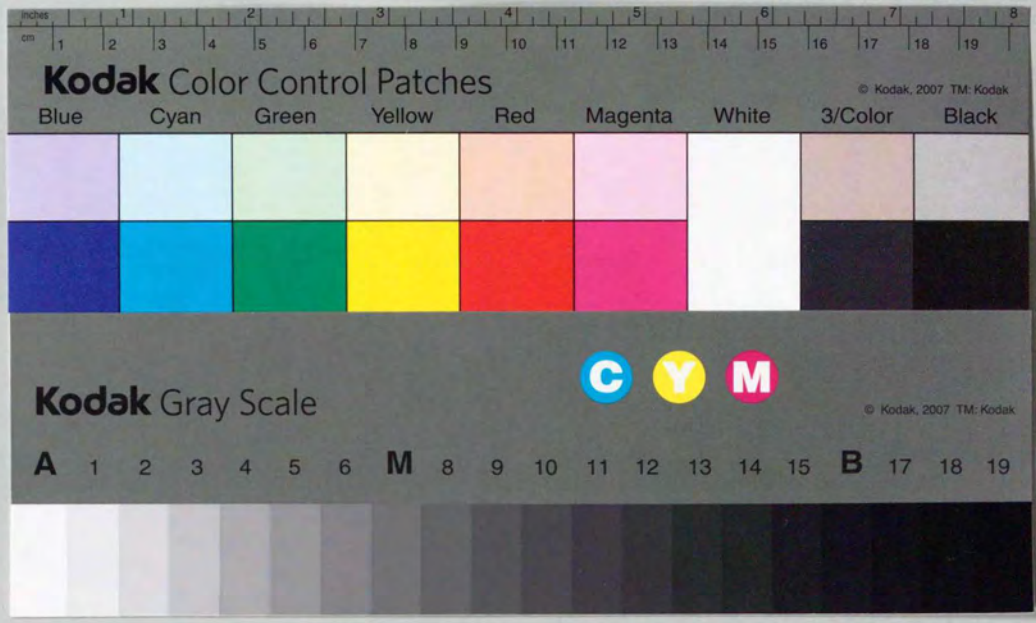
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